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I. Work Summary

The start of the project was delayed for several months because we moved our laboratory to larger and better facilities in the new MGH research building in August 1989. This slowed real forward progess until the fall. Since then, I believe we have made considerable progress.

A. Purification of LPS binding substances in inflammatory serum

We radiolabeled E. Coli 018, E. Coli 0113, E. Coli 0111:B4 and, S. typhimurium by growing the organisms in tritated broth and extracting the ^{3}H-LPS by the hot phenol method as described in the original grant protocol. We also prepared and aliquotted large quantities of normal and tolerant (acute phase) rabbit sera. We prepared affinity purified anti-LPS IgG by coupling LPS to epoxy-linked sepharose beads, and in turn coupled the anti-LPS IgG to sepharose beads using cyanogen bromide to prepare columns capable of affinity purifying LPS (and whatever is bound to it) from normal and tolerant sera. In later experiments we have utilized a murine Mab directed to the O-polysaccharide antigen of E. Coli 0111:B4 to prepare similar affinity columns. We incubated unlabeled and/or labeled LPS in each sera, affinity purified the complexes, eluted them with KSCN, and then analyzed the eluted material on SDS polyacrylamide gels. To condense much work into a single summary, we have identified a somewhat elusive band of 27KD which has been present in some but not all preparations. In the most recent experiments have had difficulties with non-specific binding, confounding our results. A problem is that the experiments are long due to the copious washing of the column that is needed, the need to concentrate the elutions by lyophilization, and the need to have multiple appropriate controls including normal serum and the use of heterologous LPS. Accordingly, we are planning to switch our experiments to the HPLC system. At this point we are uncertain regarding the reality of the band, but we are optimistic, and plan to continue this approach aggressively. Our clearest and most consistent results were generated in the early experiments using affinity columns prepared with monoclonal antibody. We have found that the columns lose efficiency after multiple elutions resulting in more non-specific binding. We are therefore in the process of preparing new affinity columns using larger quantities of monoclonal anti-O polysaccharide IgG.

In a different approach, again as outlined in the original grant, we have employed a radioimmunoassay which utilizes 1.1% dextran and 0.4M $CaCl_2$ to precipitate complexes of LPS and lipoprotein (LPS-LP) formed in normal and tolerant sera. Unbound LPS does not precipitate in the system. Using this assay and our new reagents we have confirmed that LPS binds much more rapidly and to a greater extent in the tolerant sera. We have further defined the kinetics of this binding in vitro and in vivo. The binding of LPS to lipoprotein is extremely rapid in tolerant serum. By one minute 47.0% of

^{3}H-LPS from E.Coli 0113 is bound to lipoproteins in rabbits made tolerant to LPS compared to 27.0% in normal rabbits. I am in the process of writing these results up for publication. We have purified the lipoproteins from normal and tolerant sera by ultracentrifugation at density $\leq$ 1.21 g/ml. By adding these preparations into normal serum and using the radioimmunoassay, we have confirmed that the factor responsible for the increased binding is found at density $\leq$ 1.21 g/ml; i.e.,it is found in the lipoprotein density range. To further purify the substances we passed purified lipoproteins from tolerant serum over a 1 meter column containing sepharose 6B-Cl. Four peaks were obtained, of which the most activity was found in the third peak. We are in the process of repeating this experiment. For subsequent steps we plan to use the HPLC system which will speed up the work.

B. Binding of ^{3}H-LPS to substances in polyclonal serum to E. Coli J5

Although not written into the initial grant, we were curious as to whether some or all of the protection offered by the passive transfer of antiserum raised to E. Coli J5 could be explained by an increased binding of LPS to lipoproteins. Accordingly we compared the binding of ^{3}H-LPS to lipoproteins in normal serum with that in polyclonal rabbit serum raised to E.Coli J5 using ultracentrifugation of ^{3}H-LPS incubated in these sera at density 1.21 as well as the radioimmunoassay described above. We found that there was no increased binding of ^{3}H-LPS to lipoproteins in this antiserum, but that more ^{3}H-LPS was precipitated by Ca^{++}/dextran in the antiserum. The precipitation of ^{3}H-LPS was removed by delipidation, restored by greater than 1% fresh normal sera, and was due to IgG in the antiserum. Purified IgG was unable to precipitate heterologous ^{3}H-LPS in the absence of normal serum. These finding have been highly reproducible, are true for both murine and lapine polyclonal antisera to E. Coli J5, and hold for 3 different heterologous ^{3}H-LPS.

The most logical explanation for our findings is that the LPS undergoes a physicochemical change in the presence of normal serum constituants, allowing IgG to bind to it more efficiently. Presumeably the change in conformation exposes epitopes that are ordinarily hidden in core region of LPS, although we have not yet ruled out the possibility that small mitogenic increases in anti-O polysaccharide IgG are playing a role. I believe that these findings, although not the main goal of our grant, are very important. There is currently no known in vitro test which measures the cross-reactivity of immunoglobulin in this antiserum, and no test which correlates with the protective efficacy of this antiserum. The need for normal serum in the reaction mixture may explain why solid phase immunoassays such as ELISA and western blotting methods have not been successful for measuring this binding, and suggests that a fluid phase immunoassay in the presence of at least 1% normal serum would be preferable. An article describing these results is appended to this report.

C. Interactions of different forms of LPS with macrophages

We also have extended our previous finding concerning the very different interaction of LPS and LPS-LP at the macrophage surface. We prepared complexes of LPS-LP by ultracentrifugation using ^{3}H-LPS from E. Coli 018, and adjusted the concentration of the ^{3}H-LPS in each form to be identical. In collaboration with Dr. Jean Marc Cavaillon of the Pasteur Institute in Paris,

France, we compared the ability of the two forms of LPS to induce cytokines from murine macrophages and human circulating monocytes. LPS in the LPS-LP form was 100-1000 fold less potent in inducing TNF, IL-1 and IL-6. We found, using both direct and competition binding assays, that ^{3}H-LPS-LP is unable to bind to the macrophage surface, as opposed to ^{3}H-LPS. In contrast, LPS and LPS-LP were similar in their ability to induce a mitogenic response in murine splenocytes. These results were published this month and a copy of the article is appended.

In other experiments, we studied the kinetics of the production of endogenous pyrogens and LAF (IL-1) following exposure of lapine peritoneal macrophages to LPS or LPS-LP. LPS or LPS-LP were incubated with 10^{7} macrophages for different time periods from seconds to 2 hours, followed by extensive washing by centrifugation and further incubation for 18 hours. The macrophage supernatants, which were LPS-free by limulus lysate testing, were then injected into rabbits to test for pyrogenic activity and tested for IL-1 using the mitogenic response of splenocytes from C3H/HeJ mice in the presence of concanavelin A. These results indicated that several seconds exposure to unbound LPS were enough to commit the macrophage to produce endogenous pyrogens and IL-1, whereas it took larger doses and at least 30 minutes of in vitro exposure to LPS in the LPS-LP form to see any response at all. These results are being written up for publication and will be appended in manuscript form to the Sept 31, 1990 progress report.

We also followed our plan outlined in the original grant proposal to develop an in vitro assay of TNF production by the RAW 267.4 murine macrophage cell line. The assay is straightforward but time consuming. Cultured RAW 267.4 cells were exposed to LPS incubated in different sera. The supernatants were then assayed for TNF by monitoring cytolysis of the fibroblast 929 cell line in the presence of actinomycin D. An advantage of such a system is that it measures the functional ability of LPS complexes formed in different sera to interact with cell membranes, a process that we believe is very important to understand. In early experiments we generated a dose response curve to unbound LPS. Then, in a series of experiments we preincubated LPS from different gram negative strains in normal or tolerant rabbit serum. We diluted each serum-LPS mixture 1:100 and cultivated the macrophages with this solution (LPS concentration 10 ng/ml or 100 ng/ml at the cell level, final serum concentration 1%). LPS in tolerant serum consistently induced less TNF. We also studied LPS incubated in polyclonal rabbit antiserum to E. Coli J5 in the system. This antiserum also blunted the TNF response, especially to LPS from certain gram negative strains, although these data were less consistent and are still preliminary.

II. New Knowledge

1. Tritiated LPS from three smooth strains of gram negative bacteria bind more rapidly and to a greater extent to lipoproteins in tolerant serum than normal serum.

2. More tritiated LPS is precipitated from normal serum spiked with lipoproteins from tolerant sera, indicating that the responsible agent for the increased binding is some sort of altered lipoprotein.

3. LPS bound to lipoprotein (LPS-LP) is 100-1000 fold less efficient in stimulating the synthesis and secretion of TNF, IL-1 and IL-6 from murine macrophages and human monocytes. Bound and unbound LPS have similar efficiency in inducing a mitogenic response from splenocytes.

4. LPS-LP does not bind to the surface of murine macrophages as assessed by direct or competition binding experiments, as opposed to identical concentrations of unbound LPS.

5. Macrophages exposed to LPS for seconds followed by removal of the LPS from the media are nevertheless committed to the production of endogenous pyrogen and IL-1 (and presumeably TNF and IL-6). In contrast, macrophages exposed to LPS-LP become committed to the production of endogenous pyrogen and IL-1 only after greater than 30 minutes of exposure at tenfold higher doses.

6. The RAW 267.4 macrophage cell line can be utilized as an in vitro assay to measure the ability of LPS to induce TNF. LPS incubated in tolerant serum induces less TNF than LPS incubated in normal serum in this system. Similarly, LPS incubated in polyclonal rabbit antiserum to E. Coli J5 induces less TNF, but these results are variable and still preliminary.

7. Tritiated LPS incubated in polyclonal antiserum to E. Coli J5 behaves differently than in tolerant serum. ^{3}H-LPS binds to lipoproteins in this antiserum in a similar manner as in normal serum, as assessed by ultracentrifugation. However, calcium and dextran precipitate more radiolabeled LPS from J5 antiserum compared to normal serum. The precipitation is due to IgG in the antiserum, depends absolutely upon the presence of small amounts of normal serum in the reaction mixture, and is time and temperature dependent. IgG purified from normal serum does not precipitate LPS. This reaction can serve as a fluid phase radioimmunoassay for the binding of IgG to heterologous LPS in J5 antiserum.

III. Significance

We feel that we are slowly but steadily moving along our outlined protocol in attempting to identify the substance in tolerant serum which binds increased amounts of LPS. The nature of the substance is still elusive, but we are making progress. As expected, the isolation is proceeding more slowly than we would expect for an uncomplicated protein because the substance is a lipoprotein (or lipoprotein-like) and consequently established isolation procedures are rudimentary. In addition several of our procedures are very time consuming. We are, however, about where we expected to be in our original time table. We expect that the transition to HPLC will allow us to go much faster.

In the process of our experiments, we are learning about the interactions of LPS in different sera, and also about the interactions of these different physicochemical complexes of LPS with macrophages. Although this aspect of our studies was not written into our original grant, our findings seem increasingly important and are relevant to several strategies which are being pursued to treat endotoxemia. Collectively, our findings over the year together with prior knowledge suggest the following hypothesis: After LPS enters the bloodstream, it undergoes a transformation and then binds to substances at density < 1.21 g/ml, i.e., in the lipoprotein density range. If there has been prior exposure to even small amounts of LPS, this reaction proceeds much more rapidly. The agent responsible for the increased binding is an altered lipoprotein-like particle present in inflammatory (acute phase) serum. The product of this reaction (LPS-LP) is much less toxic, presumably at least in part because LPS in the complex does not bind to macrophages, and does not induce cytokines. LPS that is not bound to lipoproteins binds to the macrophage membrane. Macrophages which have come into contact with unbound LPS for very short time intervals (seconds) are committed to release cytokines, even if the LPS in the vicinity of the macrophage is subsequently cleared or bound to lipoproteins.

There are several implications of this hypothesis. First, any strategy based upon substances which bind to LPS to block the action of Lipid A (eg, anticore antibodies, acute phase reactants, polymyxin B analogues etc.) will either need to be given prophylactically or extremely rapidly following the onset of a gram negative infection, or will need to depend upon neutralizing or clearing subsequent LPS that enters the bloodstream after the substance is given. Second, since at least two forms of LPS are present in the bloodstream with markedly different biological activities, strategies should optionally be designed to deal with the toxic unbound form. (LPS binding substances which bind to LPS-LP would be much less effective.) Third, since LPS-LP circulates in the bloodstream in a relatively non-toxic form, the measurement of total LPS concentration (as is done with the limulus lysate test) would not be expected to correlate temporarily with biologic effects.

We believe our results generated with polyclonal antisera to E. Coli J5 are of considerable importance. Our findings indicate that the acute phase substance we are studying in tolerant serum is not present in this antiserum. Thus, it does not appear that increased binding to lipoproteins is a mechanism by which J5 antiserum protects. However, we somewhat fortuitously discovered a radioimmunoassay which measures binding between immunoglobulin in this antiserum and heterologous tritiated LPS. The necessity for the presence of fresh serum for LPS-immunoglobulin binding to take place suggests that this binding is dependent on or markedly increased by LPS-serum interactions which result in a physicochemical change in the LPS. This finding has three important implications. First, it may explain why solid phase immunoassays such as ELISA and western blotting systems are poor at measuring the cross-reactivity of these antisera. Second, it may lead to an in vitro test which correlates with protective efficacy. (A theoretical and practical flaw in the two large clinical studies recently completed using commercial monoclonal antibodies to core LPS determinants is the apparent lack of such a test). Third, it may lead to a means of screening for future monoclonal antibodies with better protective efficacy than those currently existing.

IV. Publications

To date, the work has resulted in a published article, an article being submitted, and a chapter on the treatment of burn injuries which is included here because of its relevance to our work and because the grant is acknowledged. These three are appended, and are listed below.

1. Cavaillon JM, Fitting, C, Haefner-Cavaillon N, Kirsch SJ, Warren HS. 1990. Cytokine Response by Monocytes and Macrophages to Free and Lipoprotein-Bound Lipopolysaccharide. Infect Immun 58: 2375-2382

2. Warren HS, Glennon M, de Deckker FA, Tello D. Role of Normal Serum in the Binding of Rabbit Antibodies Raised to E. Coli J5 and other Gram-Negative Bacteria to LPS. Submitted.

3. Warren HS, Burke JF. Infection of Burn Wounds: Evaluation and Management. In Current Clinical Topics in Infectious Diseases-11. Ed. Swartz MN and Remington JS, Blackwell, Cambridge. In press.

We are in the final stages of preparing two other articles for publication. One follows directly from the above described work, and compares the kinetics of the transformation of LPS to LPS-LP in normal and inflammatory serum in vitro and in vivo with the kinetics of macrophage response to each form. The other article describes earlier work done involving a LPS binding protein isolated from limulus lysate. It is mentioned here because it is germain to the subject of LPS neutralization and potentially to ongoing work at the Navy. The anticipated titles of these manuscripts are listed below and should hopefully be included with the September 31, 1990 progress report.

1. Warren HS, Riveau GJ, Riveau BG, De Deckker FA and Chedid LA. Kinetics of in vitro and in vivo LPS-lipoprotein binding in normal and inflammatory serum compared with time of macrophage response to bound and unbound LPS. Manuscript in preparation.

2. Warren HS, Glennon ML, Wainwright N, Kirsch SJ, Riveau GR, Whyte RI, Zapol WM. and Novitsky TJ. Binding and neutralization of endotoxin by limulus anti-LPS factor. Manuscript in preparation.

V. Relevant work by other investigators

Tobias and coworkers have described an inflammatory protein, a glycoprotein of MW 60,000, which they have called LBP for LPS Binding Protein

(J. Biol Chem 1988 263, 13479-81). This protein slows the binding of LPS from rough mutant S. Minnesota Re595 to lipoproteins in the presence of 20mM EDTA, and this property was the assay upon which the purification was based. LBP evidently potentiates the production of TNF from macrophages prepared from tolerant animals, (unpublished data).

The relationship of LBP to our studies, if any, is unclear. We do not believe that the substance that we are attempting to isolate is LBP for several reasons. First, our factor is found at density of less than 1.21 g/ml whereas LBP, being a serum protein, is found at higher density. Second, we consistently and repeatedly find increased binding of LPS from smooth gram negative strains to lipoproteins, not decreased binding. Third, our factor decreases the bioactivity of LPS. Fourth, we were unable to repeat Tobias' experiments in the absence of EDTA and with tritiated LPS from smooth gram negative organisms, although we have in the past found similar results using rough LPS in the presence of EDTA. We are concerned that the described conditions of binding of LBP (i.e., in EDTA and with rough LPS) may not be relevant to the forms of LPS released in clinical infections (smooth LPS in the absence of calcium chelators). We therefore are proceeding with our initial protocols, keeping abreast of the ongoing work with LBP.

VI. Goals for the next year

Our overall goals for year 2 do not differ substantially from those of year 1. We plan to continue to pursue our two strategies for isolating and characterizing the substances in the lipoprotein density range which bind and neutralize LPS. These consist of the affinity purification of LPS complexes formed in tolerant sera and progressive purification using chromatographic techniques and the radioimmunoassay. For both approaches, we plan to shift to HPLC following initial purification steps. This should speed up both approaches considerably in addition to increasing both resolution and sensitivity.

For the affinity purification approach, we will continue to utilize our low pressure columns with modifications to increase yield and decrease non-specific binding. We will primarily utilize monoclonal antibodies to the O antigen of LPS (instead of affinity purified polyclonal antibodies) and we will try different detergents and blocking agents in our buffers. The Mabs which we will use are directed to the O antigen of E. Coli 0111:B4 and E. Coli 018. We also, however, plan to couple the Mabs to gels for use in the HPLC system (used with relatively low pressure in the HPLC system). This should greatly increase the speed and resolution of our protocols. The format of these experiments will be similar, (i.e. we will affinity purify LPS complexes formed in normal and inflammatory sera). We will analyze the eluted complexes by SDS-PAGE. In addition, we will further purify eluted complexes by HPLC on gel filtration columns, comparing eluted peaks from normal and inflammatory sera. As with the conventional chromatography experiments, controls will include experiments performed in the absence of LPS and with LPS heterologous to the monoclonal antibody coupled to the column. We will pay particular attention to the molecular weight range around 27,000 KD where we have found a candidate protein band on SDS-PAGE of affinity purified complexes from tolerant sera.

In our other approach, we are using the Ca++/dextran precipitation radioimmunoassay to progressively purify the substance in the lipoproteins of tolerant sera which bind LPS. Using ultracentrifugation and conventional chromatography of the lipoproteins on sepharose 6B-Cl, we have obtained 4 peaks, one of which has considerable activity. We plan to repeat these experiments and use this peak as a starting material to further purify the substance by HPLC. We plan to try columns based on hydrophobic interactions first to capitalize on the fact that the factor is lipophilic. Analysis will be by SDS-PAGE. We also plan to intermittently check the ability of the substance to neutralize LPS in the limulus assay and the TNF assay described above and in the initial protocol.

VII. Other potential goals

Our foremost goal is to isolate and characterize the substance in inflammatory serum which binds and neutralize LPS. Thus we plan to concentrate almost entirely on this main goal, which should lead to the direct benefit of providing a potentially therapeutic material for endotoxemia.

However, our studies of the different physiochemical forms which LPS takes in inflammatory serum, the different binding and stimulation of macrophages by these forms of LPS, and the binding of IgG from J5 antisera to LPS incubated in normal serum raises some provocative questions that at some point should possibly be addressed. For example, does IgG from J5 antisera bind equally to LPS and LPS-LP? Do the complexes of IgG and LPS formed in the presence of normal serum bind to the macrophage surface? Do these complexes stimulate cytokine production? What complexes are formed when IgG from J5 antiserum is added to inflammatory serum containing LPS? (This latter question seems particularly important since J5 antiserum, or an anti-core Mab, will be administered to patients in shock who probably will have already been exposed to small amounts of LPS). Does IgM isolated from J5 antiserum behave similarly? (Preliminary experiments suggest that it does). Do the Mabs to the LPS core which are currently being developed bind in a similar way, and if so, does protective efficacy correlate with binding?

Each of these questions is beyond the scope of our original grant, and thus we do not have specific plans to address them over the next year. However, our data may be relevant to other ongoing studies supported by the Navy.

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Cytokine Response by Monocytes and Macrophages to Free and Lipoprotein-Bound Lipopolysaccharide

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Recent evidence suggests that bacterial lipopolysaccharide binds to serum lipoproteins in vitro and in vivo and that lipopolysaccharide in the form that is bound to lipoprotein is less biologically active in several experimental models. In order to study the mechanism of this apparent detoxification, we compared the ability of free and lipoprotein-bound lipopolysaccharide from *Escherichia coli* O18 to stimulate interleukin-1, interleukin-6, and tumor necrosis factor from elicited murine peritoneal macrophages and circulating human monocytes. Lipopolysaccharide bound to lipoprotein was 20- to 1,000-fold less active than the unbound form in inducing the release of each cytokine. We also studied the binding of each form of lipopolysaccharide to the macrophage surface. Lipopolysaccharide complexed to lipoprotein was unable to compete for the binding of radiolabeled heterologous lipopolysaccharide to murine macrophages, and radiolabeled lipopolysaccharide-lipoprotein complexes bound poorly compared with molar equivalents of free lipopolysaccharide. Our experiments suggest that in the process of binding to lipoproteins, lipopolysaccharide may be rendered less toxic through a mechanism of decreased ability to induce monocytes and macrophages to release cytokines, perhaps because of an altered interaction at the cell surface.

The role that bacterial endotoxin (lipopolysaccharide [LPS]) plays in the pathogenesis of severe gram-negative infections has been studied for over 50 years. The majority of these investigations have utilized LPS that has been chemically extracted and then suspended in aqueous buffers. Over the last decade, however, it has been shown that LPS binds to lipoproteins (LP) in serum and plasma (15, 16, 28, 29) and that the resulting LPS-LP complex is much less active than unbound LPS in numerous assays of biological activity, including LPS-induced fever (16, 28), neutropenia (28), thromobocytopenia (29), complement activation (28), rate of development of hypotension (12), and death in adrenalectomized mice (28). Other studies have shown that the formation of LPS-LP complexes also occurs in vivo (16) and that LPS contained in membrane fragments binds to LP in a similar manner (16).

There is considerable evidence that the binding of LPS to LP is altered in inflammatory serum. Tobias et al. (22–25) have reported that the binding of LPS from rough mutant *Salmonella minnesota* Re 595 to LP is slower in inflammatory serum than in control serum. The delay is due to the formation of an intermediate complex of the LPS with a glycoprotein with a molecular weight of 60,000 called LPS-binding protein. Moreau and Skarnes, however, suggested in 1973 that LPS derived from smooth organisms incubated in tolerant rabbit serum underwent rapid transformation into a less toxic complex that contained LP (13). We have confirmed that two other LPS derived from smooth organisms, *Escherichia coli* O113 and *Salmonella typhimurium*, bind much more rapidly to LP in sera prepared from rabbits made tolerant to LPS (32) or in serum prepared from blood drawn after a single injection of interleukin-1 (IL-1) (33) than in normal serum. These experiments suggest that there are inducible humoral mechanisms that may function to decrease the bioactivity of smooth LPS by modulating LPS-LP binding (13, 18, 32, 33).

Although two studies have indicated that the structure of LPS within the LPS-LP complex remains intact (15, 28), there is little known about the mechanism(s) of the relative decrease in biological activity of the bound form. We previously reported that LPS-LP is less potent than unbound LPS in stimulating rabbit peritoneal macrophages to produce IL-1 (33). These results raised several questions concerning the interaction of LPS with macrophages. First, it was unclear whether the decrease in IL-1 secretion represented a decrease in IL-1 production or normal IL-1 production with a decrease in IL-1 release, as has been recently described for LPS contained in liposomes (1). Second, it was unclear whether the secretion of both IL-1α and IL-1β is decreased and whether the release of other cytokines, such as tumor necrosis factor (TNF) and IL-6, is similarly decreased. Third, in our previous report we utilized only elicited peritoneal macrophages from rabbits, and it was not known whether macrophages from different species and different sources would behave in the same manner. Since injected LPS that is converted to the LPS-LP form is cleared from the bloodstream relatively slowly, with a half-life of 12 h (12), a relevant question was whether the LPS-LP could stimulate circulating monocytes. A final question was whether the LPS that is bound to LP is less able to bind to the macrophage surface.

In order to address these questions and in light of recent reports suggesting that cytokines such as IL-1, TNF, and IL-6 may play a direct role in the pathogenesis of endotoxic shock (2, 3, 26, 31), we studied the interaction of the two forms of LPS with murine peritoneal macrophages and human circulating monocytes. We initially studied the release of IL-1, TNF, and IL-6 in response to LPS and purified LPS-LP. These findings confirmed that LPS-LP was less able to stimulate release of each cytokine and that the

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decrease in IL-1 resulted from a decrease in production rather than a block in secretion. We then employed a competitive-binding assay with radiolabeled LPS from *Neisseria meningitidis* to estimate the relative binding of each form of LPS to the macrophage surface (6). We found that LPS-LP cannot compete with *N. meningitidis* LPS for binding to macrophages, suggesting that its decreased activity may result from an altered interaction with the macrophage surface. Direct-binding experiments confirmed this finding. Our results are complementary to a study published after we had initiated our experiments suggesting that the release of IL-1, TNF, and IL-6 from human monocytes by LPS is diminished by prior incubation of the LPS with human serum and that this inhibition is due to LP (8).

MATERIALS AND METHODS

Preparation of LPS. A culture of *E. coli* O18K− was the kind gift of H. Williams Smith (Houghton Poultry Station). Biosynthetically radiolabeled *E. coli* O18 LPS was prepared by growing the organism in the presence of [^{3}H]acetate followed by hot-phenol extraction according to a modification of the procedure of Rudbach et al. (20) as previously described (32). Briefly, we grew cultures of *E. coli* O18 to an optical density of 0.9 at 540 nm in tryptic soy broth in the presence of 10 mCi of [^{3}H]acetate. The cells were chilled and washed three times in saline, and then the LPS was twice extracted at 65°C by using the hot-phenol method. The resulting preparation was exhaustively dialyzed against water and then treated with DNase, RNase, and protease as described by Romeo et al. (19). The concentration of LPS was estimated by a spectrophotometric *Limulus* lysate gelation assay utilizing an *E. coli* O113 LPS standard containing 10 endotoxin units/ng (lot 20; Associates of Cape Cod, Falmouth, Mass.) (17). A solution adjusted to 1 μg of LPS biological activity per r[illegible]ontained 6,150 cpm/μg when a 0.4-ml volume was co[illegible] with 4 ml of Optifluor scintillation fluid (Packard, Downers Grove, Ill.). In prior experiments using this technique for the intrinsic labeling of LPS, greater than 99% of the radiolabeled LPS was demonstrated to remain in the water phase after a 1:1 ether-water extraction at pH 5, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by autoradiography resulted in a regularly spaced band pattern typical of LPS. Unlabeled LPS from *E. coli* O18 was prepared in an identical manner from bacterial cells that were grown in the absence of [^{3}H]acetate.

LPS-LP was produced essentially as described by Ulevitch and Johnston (28). Radiolabeled *E. coli* O18 LPS was diluted 1:39 in unlabeled *E. coli* O18 LPS and was then incubated at concentrations of 60 or 250 μg/ml in 4 ml of normal rabbit serum made 20 mM with EDTA for 3 h at 37°C. Sufficient KBr and water was then added to adjust the volume to 10 ml and the density to 1.21 g/ml, and this solution was centrifuged in two 5-ml ultracentrifuge tubes for 48 h at 225,000 × *g*. The top one-third of each tube was then collected. Under these conditions, most of the LPS is found within the lower-density LP fractions at the top of the tube (32). Control unbound LPS was made by incubating and centrifuging identical quantities of radiolabeled LPS in saline made 20 mM with EDTA, followed by collection of the bottom one-third of the tube. Each fraction was then dialyzed exhaustively against pyrogen-free saline and adjusted on the basis of counts per minute to the concentrations indicated. A similar procedure was utilized to prepare a small amount of undiluted [^{3}H]LPS-LP from ^{3}H-labeled *N. meningitidis* which was radiolabeled as described below. Control LP not complexed to LPS were made by using the same techniques but adding pyrogen-free water in place of LPS. Preparation of LPS, LPS-LP, and LP were done at the same time in parallel.

Extrinsically radiolabeled LPS from *N. meningitidis* was the kind gift of Martine Caroff (UA Centre National de la Recherche Scientifique 1116, Orsay, France). We used this LPS for competition and direct-binding experiments as previously described in order to achieve the high specific activity necessary to measure binding (10). This LPS had been extrinsically radiolabeled by a modification of the procedure of Watson and Riblet (34). Briefly, 2 mg of the endotoxin was suspended in 500 μl of $NaIO_4$ solution (2×10^{-2} M), and the mixture was kept for 20 min at room temperature. Then 5 μl of ethylene glycol (5 M) was added. After 15 min at room temperature, the suspension was centrifuged at 290,000 × *g*. The pellet was suspended in 100 μl of ice-cold water and treated for 1 h at 4°C with $NaB[^3H]_4$ (2.7 mCi; specific activity, 1 Ci/mmol) in 400 μl of borate buffer (0.05 M; pH 9); after addition of 100 μl of a solution of $NaBH_4$ in water (5 mg/ml), the mixture was kept overnight at 4°C. Five hundred micrograms of $NaBH_4$ was added. After 1 h at 4°C, excess sodium borohydride was destroyed with 5 μl of acetic acid. After centrifugation for 45 min at 290,000 × *g*, the pellet was suspended in water and lyophilized. The specific activity of the endotoxin preparation was 75,000 cpm/μg. A recent observation indicated that the radiolabeled *N. meningitidis* LPS was a mixture of two forms with different ratios of binding to cell membranes. *N. meningitidis* [^{3}H]LPS with a high binding efficiency was obtained following further separation of both forms in an isobutyric acid-ammonia mixture (unpublished data). Either this isolated form or the initial preparation was used in the binding experiments.

Preparation of human monocytes. Peripheral blood mononuclear cells were obtained by centrifugation on Ficoll (MSL; Eurobio, Paris, France) of 1:2 diluted heparinized venous blood from healthy adult volunteers. Monocytes were selected by allowing the mononuclear cells to adhere to plastic culture dishes (24 wells; Nunc, Rosklide, Denmark) in the absence of serum (11). In these conditions, more than 85% of the adherent cells were monocytes, as assessed by morphological analysis by phase-contrast microscopy, histochemical staining for nonspecific esterase activity (27), and indirect immunofluorescence staining using anti-lymphocyte antibodies, OKT11 (Ortho Diagnostics, Inc., Raritan, N.J.) and IOBI (Immunotech, Marseille, France).

Mouse peritoneal macrophages. BALB/c mice (Institut Pasteur, Paris, France) of either sex, 2 to 3 months old, were injected intraperitoneally with 1.5 ml of thioglycolate medium (Diagnostic Pasteur, Paris, France); 5 days later, the peritoneal cavities were washed twice with 2 ml of RPMI 1640 medium containing 2 IU of heparin per ml. Macrophages were purified from peritoneal exudate cells by surface adherence as previously described (5). As judged by nonspecific esterase staining, more than 85% of the adherent cells were macrophages.

IL-1 induction and IL-1 assay. Human mononuclear adherent cells (5×10^5 nonspecific esterase-positive cells per well) cultured in RPMI 1640 medium (GIBCO) without serum, supplemented with 100 IU of penicillin per ml and 100 μg of streptomycin per ml were incubated for 24 h in the presence or absence of IL-1 inducers. The culture supernatants were collected and centrifuged at 3,000 × *g* for 15 min. IL-1 activity found in the supernatant will be referred to as

extracellular or released IL-1. The adherent cells were lysed by three freeze-thaw cycles in 0.5 ml of fresh RPMI 1640 medium. The lysates were centrifuged at 3,000 × *g* for 15 min; the IL-1 activity found in the supernatants will be referred to as intracellular IL-1 activity. IL-1 activity was determined by [^{3}H]thymidine uptake by C3H/HeJ mouse thymocytes in the presence of a suboptimal dose of concanavalin A (0.075 μg per well) as described previously (11). The standard deviation of the results from triplicate thymocyte cultures did not exceed 18%. Results are presented as [^{3}H]thymidine incorporation. IL-1α and IL-1β were quantified by using a radioimmunoassay and enzyme-linked immunosorbent assays (Amersham, les Ulis, France, and Cistron Biotechnology, Pine Brook, N.J., respectively).

TNF assay. TNF activity was estimated as follows: 3×10^4 L929 fibroblasts in 0.1 ml of RPMI 1640 medium supplemented with 10% fetal calf serum were cultured overnight in 96-well flat-bottomed Falcon microtiter plates (Becton Dickinson Labware, Oxnard, Calif.). The next day, serial dilutions of test supernatant were added in the presence of actinomycin D at a final concentration of 2 μg/ml. Plates were incubated at 37°C for 18 h; then cells were washed and stained with crystal violet (50 μl; 0.1% in 20% methanol—H_2O) for 20 min at room temperature. The microtiter plates were rinsed gently in saline, cells were solubilized in 1% sodium dodecyl sulfate (100 μl), and dye uptake was calculated at A_{540} by using an automated micro-ELISA autoreader (Titertek Multiscan; Flow Laboratories, Inc., McLean, Va.). One unit of TNF activity was defined as the amount required to lyse 50% of L929 target cells. The assay was standardized with a human recombinant TNF-α preparation containing 2.3×10^6 U/mg of protein, kindly provided by Rhone-Poulenc (Vitry-sur-Seine, France). Titrations were performed in triplicate, and the standard deviations of the means did not exceed 20%. Activity of the assay could be inhibited by a monoclonal anti-TNF-α antibody.

IL-6 assay. IL-6 activity was determined by using the factor-dependent 7TD1 mouse-mouse hybridoma, which was the kind gift of J. Van Snick (Brussels, Belgium). IL-6 activity was assessed as described by Van Snick et al. (30) with minor modifications. Briefly, cells (2,000 per well; 96-well microtiter plates) were grown in 200 μl of RPMI 1640 medium supplemented with antibiotics, 2-mercaptoethanol (5×10^{-5} M), and 10% fetal calf serum, in the presence of serial dilutions of supernatants from stimulated macrophages. The potency of the samples was monitored by [^{3}H]thymidine uptake after 4 days of culture. [^{3}H]thymidine (0.25 μCi per well) was added 18 h before the end of the culture. One unit of IL-6 corresponds to half-maximal growth of the hybridoma cells. Recombinant human IL-6 (Genzyme, Boston, Mass.) was used as a positive control. Titrations were performed in duplicate, and the standard deviations of the means did not exceed 20%.

Direct- and competition binding assays. Assays were performed as previously described (10). Briefly, plated monocytes or peritoneal macrophages (8×10^5 cells per well) were incubated in each experiment with [^{3}H]LPS from *N. meningitidis* alone and 10 μl of normal human serum (final concentration, 4%). The residual binding measured in the presence of a large excess of nonradioactive endotoxin was taken to represent nonspecific binding; specific binding of LPS was defined as the difference between total and nonspecific binding. Since the binding of [^{3}H]LPS increases as a function of time, reaching a maximum after 45 min at 22°C (data not shown), all experiments were performed at 22°C with an incubation period of 45 to 60 min. Direct-binding assays compared binding of [^{3}H]LPS and [^{3}H]LPS-LP. For competition experiments, the cells were incubated either with 0.2 μg of [^{3}H]LPS from *N. meningitidis* alone or with [^{3}H]LPS plus a 10-fold concentration of *N. meningitidis* LPS or *E. coli* O18 LPS or LPS-LP. After washing to remove the unbound material, the [^{3}H]LPS bound to the membrane was solubilized in a sodium dodecyl sulfate-EDTA mixture (1% sodium dodecyl sulfate, 20 mM EDTA). The solubilized radioactive material was then mixed with Instagel (Packard) and measured by liquid scintillation counting. Each experiment was performed in triplicate, and each set of experiments was performed at least three times.

Mitogenicity. Stimulation of BALB/c or C3H/HePas mouse splenocytes was carried out as described previously (4). Briefly, 5×10^5 cells per well (96-well microplates; Nunc) were cultured in RPMI 1640 medium (supplemented with antibiotics and 2% fetal calf serum) in the presence of varied concentrations of *E. coli* O18 LPS or LPS-LP for 48 h at 37°C in an atmosphere of 7% CO–93% air. Seven hours before harvesting, 0.25 μCi of [^{3}H]thymidine was added per well. Thereafter, the cells were harvested on glass-fiber filters with a cell harvester (Skatron). Thymidine incorporation was determined by liquid scintillation counting.

RESULTS

Induction of IL-1 by LPS and LPS-LP. IL-1 activity was found in the cell supernatants, on the cell membranes, and in the cell lysates of elicited murine peritoneal macrophages stimulated with both LPS and LPS-LP. However, LPS was 20- to 100-fold less active in the LPS-LP form than in the unbound form (Fig. 1). Similar results were obtained with resident peritoneal macrophages (data not shown). When human circulating monocytes were studied, there was no IL-1 production or release induced by LPS-LP from human circulating monocytes even at the highest concentration studied (2 μg/ml), indicating a decrease in activity of at least 1,000-fold compared with that with unbound LPS (Fig. 2). Measurement of IL-1α and IL-1β in these cell supernatants and lysates by radioimmunoassay was consistent with the results of the biological assays for each form of IL-1, although the magnitude of the difference was somewhat less (Table 1). In both the human and mouse systems, the addition of free LP and LPS-LP to the LPS-macrophage incubations did not diminish the production or release of IL-1, confirming that serum components are necessary for the formation of LPS-LP (15) and indicating that LP do not directly or indirectly inhibit the stimulation of IL-1 by LPS (data not shown).

Induction of TNF by LPS and LPS-LP. The TNF activity in cell supernatants of elicited murine peritoneal macrophages stimulated with LPS-LP was markedly reduced in comparison with that with unbound LPS (Table 2; experiment performed twice). When human circulating monocytes were studied, free LPS induced TNF at concentrations of 2.0 to 200 ng of LPS/ml, whereas there was no TNF detected when the cells were stimulated with LPS-LP (Table 3; experiment repeated five times).

Induction of IL-6 by LPS and LPS-LP. LPS-LP induced markedly less IL-6 activity compared with LPS alone from murine peritoneal macrophages (Table 4) and circulating human monocytes (Table 5; experiment repeated three times).

LPS-macrophage binding. As described in Materials and Methods, we utilized a competitive-binding assay (10) to evaluate the interaction of LPS and LPS-LP with the murine

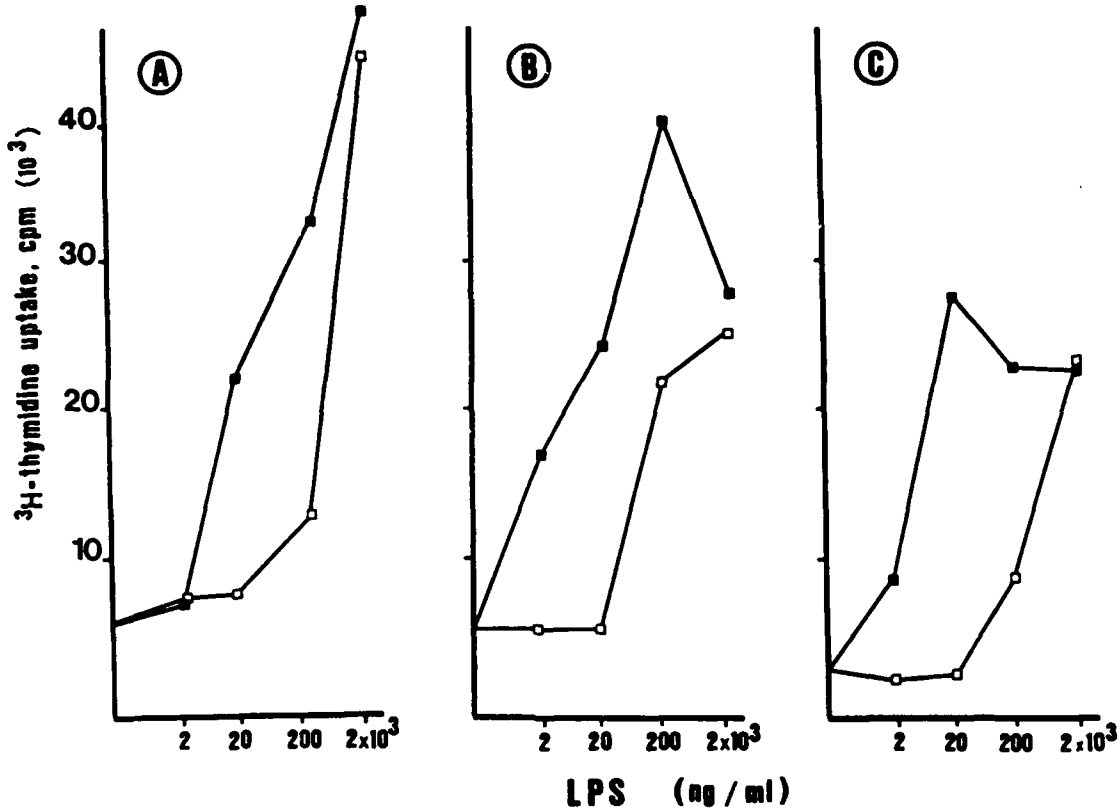

FIG. 1. IL-1 activity in cell supernatants (diluted 1:10) (A), in cell lysates (1:10) (B), or associated with the cell membrane (C) of elicited peritoneal macrophages from BALB/c mice triggered by increasing amounts of free *E. coli* O18 LPS (■) and *E. coli* O18 LPS-LP (□). Results are expressed as [^{3}H]thymidine uptake (counts per minute) by mouse C3H/HeJ thymocytes in the presence of suboptimal doses of concanavalin A and cell supernatants or cell lysates (1:10). Standard deviation did not exceed 18%. This experiment was performed three times with similar results.

macrophage membrane. A 10-fold excess of LPS (unlabeled *N. meningitidis*, *E. coli* O18, and *E. coli* O18 in the LPS-LP form) were incubated with the macrophages, followed by the addition of extrinsically radiolabeled LPS from *N. meningitidis*. The cells were then washed and evaluated for bound counts per minute. Homologous (*N. meningitidis*) and heterologous (*E. coli* O18) LPSs were able to compete for the binding of radiolabeled *N. meningitidis* LPS. In contrast, LPS-LP did not decrease this binding (Table 6). We also directly compared the binding of [^{3}H]LPS and [^{3}H]LPS-LP

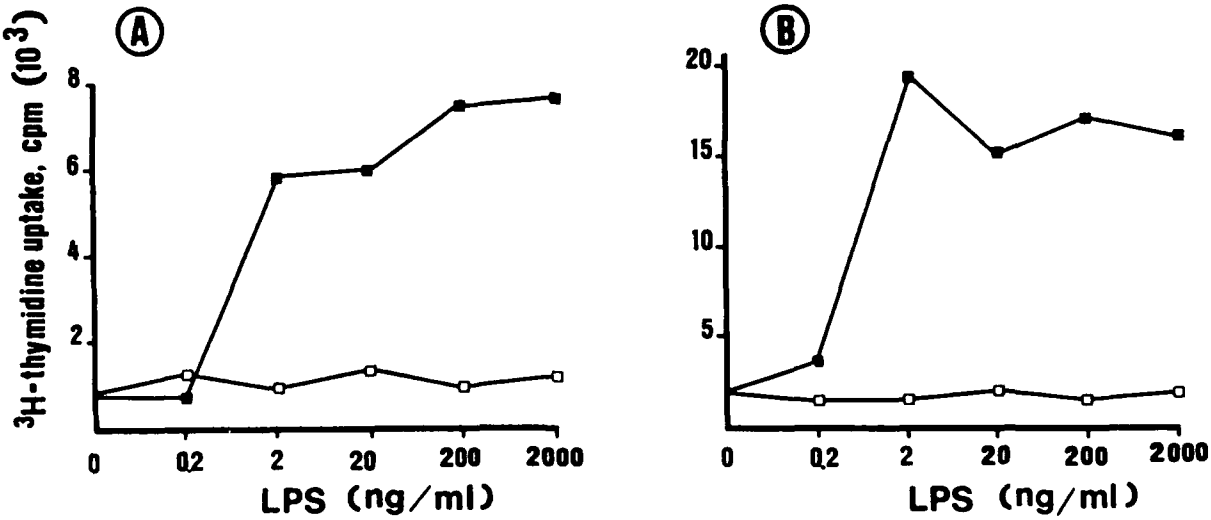

FIG. 2. IL-1 activity in cell supernatants (A) and in cell lysates (B) of human monocytes triggered by increasing amounts of free *E. coli* O18 LPS (■) and *E. coli* O18 LPS-LP (□). Standard deviations did not exceed 18%. This experiment was performed seven times with similar results.

TABLE 1. IL-1α and IL-1β produced by human monocytes triggered by LPS and LPS-LP

Inducer and concn (ng/ml)	IL-1 concn (pg/ml)			
	IL-1β		IL-1α	
	Extracellular	Cell-associated	Extracellular	Cell-associated
None	40	80	<170	187
LPS				
2	2,160	2,466	408	3,966
20	3,373	3,575	374	4,930
LPS-LP				
2	150	140	<170	374
20	120	160	204	221

TABLE 3. TNF activity released by human monocytes

Inducer and concn (ng/ml)	TNF activity (U)	
	Donor 1	Donor 2
None	<1	1
LPS		
2	ND[a]	135
20	160	125
200	220	55
LPS-LP		
2	ND	1.3
20	<1	<1
200	<1	<1

[a] ND, Not determined.

from *N. meningitidis* to murine peritoneal macrophages. These experiments confirmed that LPS in the LPS-LP form binds poorly to the macrophage membrane.

Mitogenic assay. LPS-LP was slightly less mitogenic than unbound LPS for splenocytes from C3H/HePas mice (Fig. 3A) and had essentially the same mitogenicity for splenocytes from BALB/c mice (Fig. 3B). This experiment was repeated a total of three times with similar results.

DISCUSSION

Recent studies suggest that cytokines are important in the pathogenesis of endotoxic shock. Although the induction of IL-1, IL-6, and TNF by LPS is well known, the stimulation of these cytokines by LPS-LP has not been directly and systematically addressed. Investigation of the interaction of the LP-bound forms of LPS with macrophages seemed warranted because LPS-LP complexes may be the predominant form which circulates in the bloodstream (12) and because of recent studies which suggest that the binding of LPS to LP is modulated in inflammatory sera (22–25, 32, 33). In addition, a study published after we had initiated our experiments suggested that the release of these cytokines by monocytes is inhibited by LP in human serum (8).

We recently reported that LPS-LP is less able to induce IL-1 activity from peritoneal macrophages compared with free LPS (33). Our present studies extend this finding in several ways. First, LPS-LP was much less active than unbound LPS in stimulating the release of IL-6 and TNF from macrophages. Such a finding was not unexpected for TNF because LPS-LP is apyrogenic even in microgram quantities (16, 28), whereas TNF is a potent pyrogen (7). Second, our previous studies utilized only lapine peritoneal macrophages that were elicited with thioglycolate. Our present data indicate that LPS-LP is similarly less active in stimulating resident and elicited murine macrophages and circulating human monocytes. Third, our data indicate that both IL-1α and IL-1β are diminished in monocyte supernatants stimulated with LPS-LP compared with unbound LPS and that the decrease in extracellular IL-1 from murine macrophages reflects a decrease in production rather than a block in its release. Fourth, we found that *E. coli* O18 LPS-LP was unable to compete for the binding of a heterologous extrinsically radiolabeled LPS to the macrophage surface, whereas molar equivalents of the unbound form of *E. coli* O18 LPS did compete for binding. Direct-binding experiments utilizing LPS and LPS-LP from *N. meningitidis* confirmed that LPS-LP bound poorly to the cell membrane. Finally, in an initial attempt to compare the activation of macrophages with the activation of splenocytes, we found that LPS-LP was only slightly less mitogenic than unbound LPS.

These findings are consistent with the hypothesis that at least one mechanism by which LPS-LP is less toxic in vivo is that it is less able to induce the release of IL-1, IL-6, and TNF. Our findings are complementary to those of Flegel et al., who reported that LPS preincubated in 20% normal human serum was less active in stimulating human monocytes to produce IL-1, IL-6, and TNF compared with

TABLE 2. TNF activity released by mouse peritoneal macrophages

Inducer and concn (ng/ml)	TNF activity (U)	
	Expt 1	Expt 2
None	<5	<5
LPS		
2	<30	90
20	<30	700
200	920	2,000
2,000	9,500	480
LPS-LP		
2	<5	<5
20	<5	<5
200	84	<5
2,000	175	<5

TABLE 4. IL-6 activity released by mouse peritoneal macrophages

Inducer and concn (ng/ml)	IL-6 activity (U/ml)
None	18
LPS	
20	1,700
200	1,200
LPS-LP	
20	55
200	120

TABLE 5. IL-6 released by human monocytes

Inducer and concn (ng/ml)	IL-6 activity (U/ml)		
	Donor 1	Donor 2	Donor 3
None	<30	90	83
LPS			
2	ND[a]	ND	1,750
20	5,400	ND	2,200
200	4,650	4,500	2,000
2,000	2,600	1,700	2,200
LPS-LP			
2	ND	ND	68
20	55	ND	90
200	30	150	340
2,000	70	560	1,300

[a] ND, Not determined.

TABLE 6. Inability of LPS-LP to compete for binding or to bind directly to mouse peritoneal macrophages[a]

Unlabeled material (2 μg)	^{3}H-labeled *N. meningitidis* compound (0.2 μg)	cpm bound	Δcpm[b]
Expt 1			
None	LPS	2,715 ± 978	
N. meningitidis LPS	LPS	630 ± 252	2,085
E. coli O18 LPS	LPS	1,584 ± 617	1,131
E. coli O18 LPS-LP	LPS	2,750 ± 277	0
Expt 2			
None	LPS	5,329 ± 446	
N. meningitidis LPS	LPS	218 ± 15	5,111
None	LPS-LP	92 ± 30	
N. meningitidis LPS	LPS-LP	97 ± 21	0

[a] Experiments 1 and 2 are representative of six and three different results, respectively. cpm, Counts per minute.
[b] Relative to value for the same experiment with no unlabeled material.

controls (8). The decrease in cytokine release was not seen in delipidated serum but was found in delipidated serum reconstituted with LP prepared by ultracentrifugation. Although the degree of LPS-LP binding was not measured in this study, it seems likely that part or all of the inhibition may have been due to the formation of LPS-LP.

Of interest is a recent study in which macrophages incubated with LPS incorporated into liposomes were shown to release much less IL-1 than free LPS (1). Importantly, the intracellular and membrane-associated IL-1 were not decreased, suggesting that the production of IL-1 was normal but that the release of IL-1 did not occur (1). This was not the case with LPS-LP, in which both production and release were decreased. Our findings parallel our previous findings that gangliosides inhibit the production and release of IL-1 by macrophages (6), presumably by forming LPS-ganglioside complexes (14), which are then less able to stimulate the macrophage at the cell surface.

We hypothesize that LPS-LP is less able to stimulate the macrophage membranes because the LPS component of the complex is less able to interact with the macrophage surface. Such a notion is supported by our binding studies, although we cannot be certain that these two events are related. We have previously shown for different sorts of LPS-ganglioside complexes that the competition for LPS binding and IL-1 production and release are correlated (6). Our studies are consistent with those of Freudenberg and Galanos (9), who reported that there was less LPS internalized into murine macrophages after 24 h of cultivation if the LPS was bound to high-density LP.

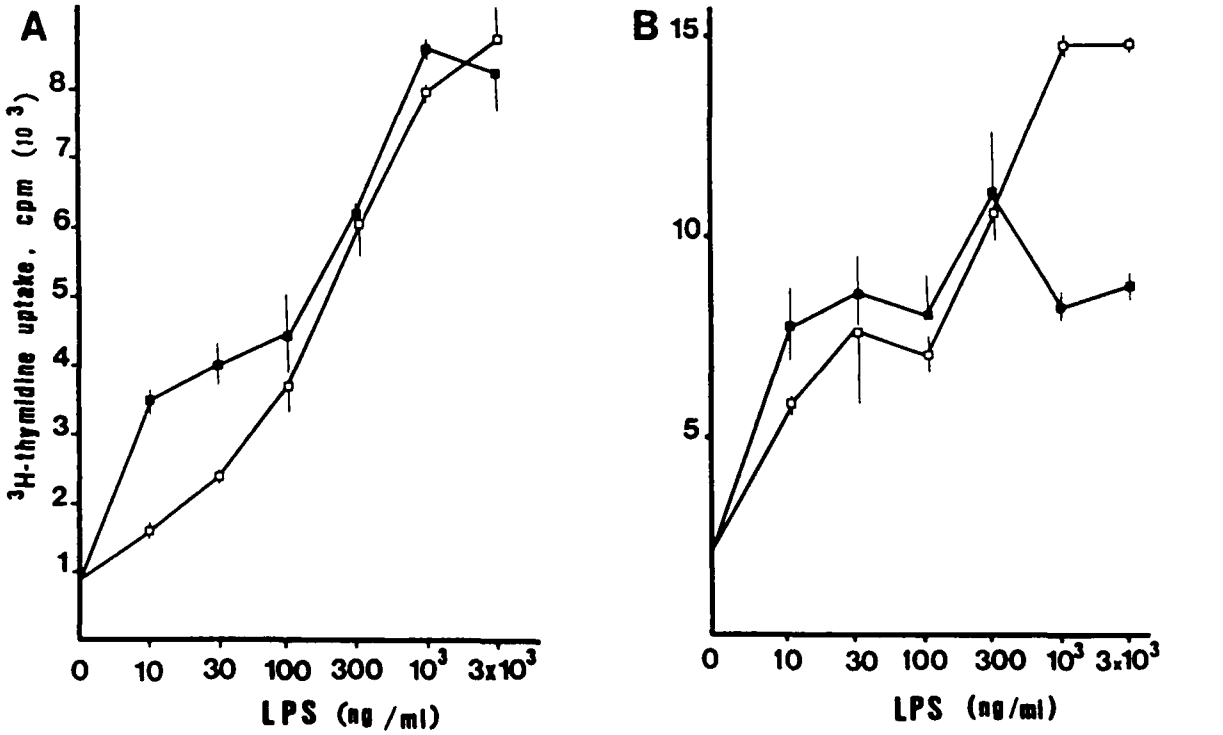

FIG. 3. Mitogenicity of free *E. coli* O18 LPS (■) and *E. coli* O18 LPS-LP (□) on splenocytes from C3H/HePas mice (A) and BALB/c mice (B). This experiment was performed four times with similar results.

Since the physicochemical forms of LPS are a critical determinant of LPS-induced cytokine release, caution needs to be taken in strictly extrapolating our findings to gram-negative infections. In this study, we utilized intrinsically radiolabeled, chemically extracted LPS from *E. coli* O18 and extrinsically radiolabeled chemically extracted LPS from *N. meningitidis* for the binding assays, a procedure which was necessary in order to have a high enough specific activity in order to measure LPS-cell binding. Chemically extracted LPS does not occur naturally and indeed Tesh and Morrison have recently shown that radiolabeled LPS from *E. coli* O111:B4 released from the bacterial surface in the presence of serum has physicochemical and biological properties different from those of chemically extracted LPS (21). On the other hand, Munford et al. reported that LPS released as membrane fragments into culture supernatants interact with LP in a manner similar to that of chemically extracted LPS, providing some reassurance that our findings are not artifactual (16).

Our observation that LPS-LP was only slightly less mitogenic than LPS is curious when contrasted with the marked difference in the two preparations in stimulating macrophages. It is possible that this finding represents the longer incubation time of each preparation with the cells (48 versus 24 h). It may be, however, that the interaction of LPS with lymphocyte membranes involves mechanisms different from those of macrophages. A similar discrepancy between mitogenicity and IL-1-inducing activity has been observed with lipid A (4, 5) and with LPS complexed to gangliosides (unpublished observation). Further work will be needed to address this issue.

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ROLE OF NORMAL SERUM IN THE BINDING OF RABBIT ANTIBODIES RAISED TO E. COLI J5 AND OTHER GRAM-NEGATIVE BACTERIA TO LPS

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Running title: Role of normal serum in LPS-antibody binding.

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ABSTRACT

Because LPS bound to lipoprotein is less active than unbound LPS in multiple assay systems, we compared the binding of radiolabeled LPS to lipoproteins in sera prepared from normal rabbits and rabbits made hyperimmune to E. coli J5. We found that LPS-lipoprotein binding in hyperimmune sera to E. coli J5 was not greater than that in normal serum as assessed by ultracentrifugation. More LPS was precipitated from hyperimmune antisera than in normal sera when the sera were combined 1:1 with 0.4M $CaCl_2$, 1.1% dextran. Radiolabeled LPS was precipitated by delipidated antisera and IgG purified by anion exchange chromatography, but the precipitation was dependant upon the presence of greater than 1% normal serum in the reaction mixture. Precipitation of heterologous LPS could be dissociated from antibody titers to E. coli J5 LPS measured by ELISA. Our data suggest that a fluid phase radioimmunoassay in the presence of normal serum may be preferable to ELISA for the detection of immunoglobulins present in antisera raised to rough mutant bacteria which bind to heterologous smooth LPS.

INTRODUCTION

Numerous investigations over the last 15 years have suggested that large quantities of passively transferred antisera raised to boiled cell vaccines of rough mutant gram-negative bacteria protect against challenge with heterologous lipopolysaccharide (LPS) or gram-negative bacteria (1-6). These studies culminated in a double blind therapeutic clinical trial in which passively transferred polyclonal antisera prepared from human volunteers vaccinated with a boiled cell vaccine of rough mutant Escherichia coli J5 was shown to significantly reduce mortality in patients with gram-negative shock (7).

It has been hypothesized that the protective element in this antisera is antibody directed to a common "core" epitope on LPS. Support for this hypothesis comes mainly from fractionation (5, 8) and absorption (1, 3, 6, 9, 10) experiments. On the other hand, the hypothesis has remained controversial for several reasons. First, some investigators have been unable to confirm that polyclonal antisera raised to rough gram negative organisms are in fact protective (11-14). Second, the majority of studies have been performed with whole antisera; the number of investigations with purified immunoglobulin have been limited because animal models require large amounts of antibody. Protection with purified immunoglobulin fractions, as opposed to antisera, has not always been found (5). Third, although absorption of the antisera with rough mutant bacteria of LPS removes both antibody and protection, it is possible that inflammatory

factors which are not antibody could be removed at the same time. Fourth, there is at present no in vitro test that correlates with protection. Development of such a test has been hampered by the fact that LPS is an amphipathic molecule whose stereochemical configuration probably depends to a great extent upon the medium in which it is suspended or the support to which it is bound. The most commonly utilized tests to measure homologous and heterologous antibody directed to LPS are enzyme immunosorbent assays (ELISA) and western blotting tests. In each of these assays LPS is fixed to a solid matrix in a manner that probably does not exist in nature and which may hide the putative protective epitope. Several monoclonal antibodies raised to rough mutant vaccines have been selected by screening with ELISA, (15-20). These antibodies bind in differing degrees to homologous and heterologous LPS or bacteria. Although one of these monoclones has been described to protect in models of gram-negative challenge (18), as well as in a very recent clinical trial (19), there appear to be multiple monoclonal antibodies that recognize E. coli J5 LPS by ELISA but which are not protective.

Because of the lack of definitive data that immunoglobulin antibody is the sole protective element in polyclonal antisera raised to rough mutant bacteria, the possibility has remained that a non-antibody inflammatory (acute phase) factor could be responsible for some or all of the protection seen. When LPS is incubated in serum, it is disaggregated and rapidly binds to serum lipoproteins to form a low density LPS-lipoprotein complex (21, 22). This complex is much less active than unbound LPS in numerous assay systems including activation of Limulus lysate (23), pyrogenicity (21, 23), induction of complement (21), induction of

leukopenia or thrombocytopenia (21, 22) and death in adrenalectomized mice (21). The binding of LPS to lipoproteins in inflammatory sera is different than in normal sera. Lipopolysaccharide extracted from rough mutant S. minnesota Re595 binds more slowly to lipoproteins in inflammatory sera because it binds preferentially to a recently described "acute phase" glycoprotein of MW 60,000 (Lipopolysaccharide Binding Protein, LBP) (24, 25). On the other hand, LPS from several smooth strains of gram negative bacteremia have been described to bind more rapidly and to a greater extent to lipoproteins in tolerant (26,27) and inflammatory sera (28). This binding to lipoprotein may provide a mechanism for the neutralization of LPS by normal sera (21-23, 29, 30), and the increased neutralization that is seen when LPS is incubated in tolerant (26, 27) or inflammatory (31) sera.

Because of the possibility that part of the protection offered by polyclonal antiserum raised to rough mutant E. coli J5 (J5 antiserum) might be mediated through altered binding of LPS to lipoproteins, we compared the binding of several smooth radiolabeled heterologous LPS to lipoproteins in this antiserum with sera prepared from normal and tolerant rabbits. In the course of these studies, we found that greater amounts of heterologous LPS were precipitated in J5 antiserum compared to normal serum under conditions designed to precipitate LPS-lipoprotein complexes. Delipidation of the antiserum eliminated the precipitation despite the maintenance of specific IgG and IgM antibodies directed to E. coli J5 LPS as assessed by ELISA. Addition of normal serum, but not lipoproteins, restored the precipitation. Since this assay was a fluid phase radioimmunoassay measuring binding between heterologous LPS and something in J5 antiserum, we investigated the phenomenon further. This article

describes these experiments, which support the concept that high concentrations of IgG prepared by anion exchange from J5 antiserum can bind to (and affect the behavior of) heterologous LPS, but that the binding is influenced by the presence of normal serum. Our data suggest that at least some of the immunoglobulins which bind heterologous LPS cannot be measured by ELISA.

METHODS

Bacteria, LPS and vaccines.

Bacterial strains E. coli 0113, E. coli J5 (Braude strain), and E. coli 018 were the kind gifts of Dr. Donald Hochstein (Office of Biologics Research and Review, National Institutes of Health, Bethesda, MD), Dr. J. Sadoff (Walter Reed Army Institute of Research, Washington, D.C.), and Dr. H.W. Smith (Houghton Poultry Station, England), respectively. The G30 mutant of S. typhimurium was the kind gift of Dr. P. Rick, (Uniformed Services University of the Health Sciences, Bethesda, MD).

Radiolabeled LPS was prepared by hot-phenol extraction (32) of bacteria which had been biosynthetically labeled with either ^{3}H-acetate (E. coli 0113, E. coli 018) or ^{3}H-galactose (S. typhimurium). The growth and extraction of labeled LPS from E. coli 0113 was performed according to the method of Rudbach (33), and has been described (27). Greater than 99% of radiolabeled LPS was demonstrated to remain in the water phase following a 1:1 ether-water extraction at pH 5. SDS-PAGE electrophoresis of this LPS followed by autoradiography resulted in a

regularly spaced band pattern typical of LPS. Radiolabeled LPS from E. coli 018 was made in an identical manner. S. typhimurium G30 mutant was specifically labeled in the polysaccharide coponent by growing the organism in medium containing ^{3}H-galactose. This organism lacks the enzyme UDP gal-epimerase and forms complete LPS only in presence of exogenous galactose (34). Radiolabeled galactose added to the culture medium is incorporated almost exclusively into the LPS (34). These organisms were grown in PPBE broth (10 g/L peptone, 1 gm/L beef extract, 5 g/L NaCl) in 0.05 mM unlabeled D-galactose and 500 uCi D-[1-^{3}H]-galactose/100 ml broth as described by Munford (35). The LPS was extracted as above except that in addition the preparation was treated with DNase, RNase and then with pronase (Sigma Chemical Co., St. Louis, MO) according to Romeo (36). Radiolabeled LPS from E. coli J5 was made by growing the organisms in tryptic soy broth medium containing ^{3}H-acetate under identical conditions as that for E. coli 0113, followed by the extraction of the rough LPS by the method of Galanos (37). The specific activities of E. coli 0113, E. coli 018, and S. typhimurium LPS were 5,200 CPM/ug, 5,600 CPM/ug, and 16,800 CPM/ug respectively where the concentration of LPS was estimated by a spectrophotometric Limulus lysate assay using E. coli 0113 LPS as a standard. The specific activity of the radiolabeled E. coli J5 was 22,000 CPM/ug by weight. Unlabeled LPS from E. coli J5 was obtained from List Biochemicals (Campbell, CA). Unlabeled LPS from E. coli 0113 was the kind gift of Dr. Thomas Novitsky (Associates of Cape Cod, Falmouth, MA). Unlabeled LPS from E. coli 018 and S. typhimurium were prepared as described above except that no radiolabel was included in the culture medium.

Killed boiled cell vaccines of each strain were prepared by boiling washed overnight bacterial cultures for 2 hr followed by further washing in saline as previously described (38). The vaccines were then dried by washing with acetone and stored at 4°C.

Rabbit sera and antisera.

Normal sera were prepared from blood pooled from six 2 kg New Zealand white rabbits. Tolerant sera were prepared from blood pooled from six rabbits 24 hr following the last of 6 daily intravenous injections of E. coli 018 LPS (day 1, 150 ng/kg; days 2-6, 1500 ng/kg) (27). Antisera to E. coli J5 were prepared from pooled blood drawn from six rabbits simultaneously immunized with bacterial vaccines and bled 7 days following the last of 9 intravenous injections of an increasing dose over 3 weeks (0.1, 0.1, 0.2. 0.2. 0.4, 0.4, 0.8, 0.8, 1.6 mg/rabbit) (38). Antiserum to S. typhimurium was prepared with the same immunization schedule. Antisera to E. coli 0113 and E. coli 018 were prepared from later bleeds from rabbits given the same immunization schedule but then given multiple booster doses over a period of months. All sera were immediately prepared, aliquotted and frozen at -70° until use.

Calcium/dextran precipitation assay.

This assay was developed to precipitate LPS complexed to lipoproteins, and has been described (28). Briefly, 0.2 mls of serum were incubated with 1.0 ug/ml ^{3}H-LPS at 37°C for 30 min unless otherwise described, followed by the addition of an equal amount of a solution of 1.1% dextran sulphate (MW 2 x 10^{6}, Pharmacia, Uppsala, Sweden) made 0.4M with $CaCl_2$. All of the serum lipoproteins precipitate in this solution

(39). The mixture was incubated at room temperature for 15 min and then centrifuged at 1200 x g for 15 mins. The resulting precipitate was dissolved in 0.2 mls 5% NaCl, diluted 1:1 with water and counted for radioactivity by adding 4.5 mls scintillation fluid (ASC II, Amersham, Buckinghamshire, England) and counting for 10 min in a scintillation counter. The supernatant was also counted and the precipitated LPS was expressed as a percentage of total CPM recovered following correction for quenching in each sample by the internal standard method. All assays were performed at least in duplicate and the results are given in means adjusted to the nearest whole percentage point. Duplicates usually did not vary by greater than two percent when the radiolabeled LPS was precipitated in this manner. In saline alone 5% of E. coli 0113, 2% of E. coli 018, 6% of S. typhimurium, and 99% of E. coli J5 LPS precipitated. When purified LPS-lipoprotein complexes were prepared by ultracentrifugation of LPS-serum mixtures in KBR at a density of 1.21 g/cm^3 (40), the percentages of lipoprotein-bound LPS that precipitated in the assay for E. coli 0113, E. coli 018, and S. typhimurium were 95%, 82% and 86% respectively.

Determination of LPS-lipoprotein binding as assessed by ultracentrifugation.

The percentage of LPS bound to lipoprotein in serum mixtures was assessed as described by Munford (40). Briefly, one ug of radiolabeled LPS was incubated with 0.5 ml serum in 5 ml ultracentrifugation tubes at 37°C. After 30 min, 4.5 ml of an iced solution of KBR was added to adjust the final density to 1.21 g/cm^3 and the tubes were ultracentrifuged at 225,000 g for 24 hr at 4°C in SW 50.1 Ti rotor. The

binding reaction between LPS and lipoprotein does not progress at 4°C (21). Each tube was then separated into the top one third and bottom two thirds by perforating the bottom of the tube with a small needle. Radioactivity in top and bottom fractions was measured by diluting a 0.5 ml aliquot 1:1 with water, adding 9 mls scintillation fluid and counting for 10 minutes in a scintillation counter. Corrections were made for quenching using the internal standard method. Lipoprotein bound LPS is expressed as the percentage of total CPM recovered that floated at density of < 1.21 g/cm^3 in the top third of the tube.

Delipidation of serum.

Sera were delipidated by three methods that preserve protein function. In the first method, the sera were extracted with a 75:25% mixture of diisopropyl ether and butanol for 3 hrs at room temperature as described by Cham (41). In the second method, the sera were agitated with the colloidic silicic acid Aerosil (Digussa Corp., Teterboro, N.J.) for 4 hrs at 45°C, followed by centrifugation to clarify the serum (42). In the third method, the sera were adjusted to a density of 1.21 g/cm^3 with KBr and were ultracentrifuged for 48 hrs at 225,000 x G. The bottom two thirds of the tube containing the lipoprotein free serum proteins were combined with a solution of KBR in water at density 1.21 g/cm^3 and recentrifuged for 24 hrs. The bottom two thirds of this tube were then exhaustively dialyzed against saline and adjusted to the original serum volume.

Preparation of immunoglobulin G.

Immunoglobulin G was purified from hyperimmune sera by precipitation in half-saturated ammonium sulphate followed by anion exchange

chromatography with DEAE Sephacel (Sigma) in 0.0175M $NaKPO_4$ buffer, pH 8.0. Flow-through protein was concentrated by ultrafiltration (model 8050; Amicon Corp., Lexington, MA) with a 30,000 MW cut-off (PM 30). The IgG was washed with normal saline adjusted to pH 7.4 in order to eliminat the phosphate ions which complexed with calcium in the precipitation assay. Protein concentration was estimated by the method of Lowry (43).

ELISA for anti-LPS antibody.

Assays for IgG and IgM class antibodies directed to the different LPS were performed in a manner similar to that previously described with minor modifications (38). Briefly, flexible polyvinyl microtiter plates (Dynatech, France) were coated overnight at $4^{o}C$ with 1 ug/ml of each LPS in 0.05M carbonate buffer, pH 9.6. The plates were then washed six times with washing buffer consisting of PBS pH 7.4 containing 0.02M $MgCl_2$ and 0.5% Tween. Dilutions of serum or IgG in washing buffer containing 0.5% BSA were incubated overnight at $4^{o}C$ and then again washed. The plates were next treated for 1 hr at $37^{o}C$ with peroxidase conjugated goat anti-rabbit IgG (Diagnostics Pasteur, France) or peroxidase conjugated goat anti-rabbit IgM, Mu chain specific (Cappel, Organon Teknika Corp, West Chester, PA) followed by incubation with O-phenylenediamine (Sigma, P3888) in citrate-phosphate buffer pH 5 for 6 min. The reaction was stopped by the addition of 12.5% H_2SO_4. Plates were read on a microtiter reader at OD_{492} and ELISA titers were obtained using a computer program to calculate the reciprocal dilution resulting in an OD_{492} of 0.25. All antibody titers were performed at least in duplicate and the results are expressed in means.

Affinity purification of anti-E. coli J5 LPS antibody.

Affinity chromatography of anti-LPS antibody was made in a manner similar as that described by Ulevitch (44). E. coli J5 LPS was coupled to epoxy-activated Sepharose 6B beads (Pharmacia) according to the manufacturers directions. The quantity of LPS that bound was estimated to be 0.46 mg LPS/gm gel by including trace amounts of ^{3}H-E. coli J5 LPS during the binding. Affinity chromatography was performed in a 12 ml plastic syringe containing 7 ml of gel in PBS pH 7.4 buffer. Two mls of antisera were diluted 1:1 with PBS, loaded onto the system and allowed to circulate in a closed loop at 4°C for 24 hrs. The column was then extensively washed for 24 hrs, and bound anti-J5 LPS antibody was eluted with 2.5M NaSCN at 4 ml/min. Eluted antibody was immediately dialyzed against 200 volumes of water, followed by exhaustive dialysis against PBS pH 7.4. The antibody was then concentrated, redialyzed briefly against normal saline adjusted to pH 7.4, and frozen at -70°C until use.

RESULTS

LPS-lipoprotein binding as assessed by ultracentrifugation and precipitation with calcium and dextran.

Radiolabeled LPS from E. coli 0113 and S. typhimurium were incubated for 30 min at 37°C in normal serum, J5 antiserum or serum from tolerant rabbits, and the mixtures were then either centrifuged in KBr at density 1.21 gm/ml or precipitated with Ca^{++}/dextran as described in Methods. These results are shown in Table I. As expected, in the tolerant serum more LPS was converted to the low density form and more of each LPS

precipitated with Ca^{++}/dextran than in normal serum (26,27). When LPS was incubated in J5 antiserum, slightly less of each LPS was converted to the low density form than in normal serum, but more of each LPS precipitated with calcium and dextran.

Because of the uncertainty of the protective element in J5 antiserum and the lack of an *in vitro* test that correlates with protection, we elected to further investigate the precipitation of the radiolabeled LPS in J5 antiserum. Although we concentrated on the differences between normal serum and J5 antiserum, in some experiments we included sera from tolerant rabbits.

Effect of incubation time and temperature on precipitation of heterologous LPS in normal serum and J5 antiserum.

E. coli 0113 LPS was incubated at 1.0 ug/ml at 37°C in normal or J5 antiserum for differing times before the addition of calcium and dextran to precipitate the LPS. These data, presented in Figure 1, show that even after 1 minute of incubation more heterologous LPS is precipitated. A similar curve was found for LPS from *S. typhimurium*. We were unable to measure the precipitation of homologous *E. coli* J5 LPS because 99% precipitated with calcium and dextran in saline alone.

When the incubation was performed at 4°C very little LPS precipitated in any of the sera (Table II).

Role of normal serum and lipoproteins in precipitation of heterologous LPS.

A leading candidate for the factor present in J5 antiserum responsible for the increased precipitation of heterologous LPS was immunoglobulin.

The assay however was designed to precipitate LPS-lipoprotein complexes. To evaluate the role of lipoproteins in the system, we delipidated normal and J5 antiserum by two methods that do not denature serum proteins and then tested the delipidated sera. (Table III). Although antibody titers directed to E. coli J5 LPS in the delipidated antisera remained relatively high, as measured by ELISA, they were slightly less than the titers in the parent antiserum. We therefore also concentrated the delipidated antisera so that the IgM antibody titer was approximately the same as in the parent antiserum. Despite the maintenance of immunoglobulin directed to E. coli J5 LPS, the difference in the percentage of precipitated heterologous LPS between normal and J5 antiserum was lost with delipidation.

It has been suggested that a common epitope present on the surface of heterologous smooth bacteria might be exposed through an interaction with normal serum (1). Accordingly, we delipidated normal serum and J5 antiserum, and tested these preparations alone and combined 1:1 with saline or freshly thawed normal rabbit serum. The addition of normal serum reconstituted the increased precipitation with J5 antiserum for each LPS (Table IV). Similar results have been found in multiple experiments using different antiserum pools and in individual rabbit antisera. These experiments suggested that the factor responsible for the increased precipitation of heterologous LPS in J5 antiserum is present in delipidated serum, but require the presence of normal serum to be manifest. The combination of delipidated antiserum and purified serum lipoproteins prepared by ultracentrifugation from either normal or J5 antisera did not reproducibly reconstitute the increased precipitation (data not shown). Delipidated tolerant serum was unable to precipitate radiolabeled LPS and the addition of normal serum to delipidated tolerant

serum did not reconstitute the increased precipitation found in fresh frozen tolerant serum.

Effect of dilution on precipitation of heterologous LPS.

Precipitation of LPS in each serum decreased rapidly with dilution in saline. Tolerant serum and J5 antiserum behaved similarly and approached normal serum at a dilution of 1/32 (Fig. 2). In all three sera there was a slight increase in the LPS precipitated at dilutions 1/32-1/256. This increase was consistently present in repeated experiments.

To assess the concentration of normal serum necessary to visualize the phenomenon, we combined delipidated normal and delipidated J5 antiserum 1:1 with dilutions of normal serum in saline and tested the mixture for the ability to precipitate heterologous LPS. These results are shown in Fig. 3.

To evaluate the concentration of the factor in J5 antiserum necessary to precipitate increased heterologous LPS, we diluted J5 antiserum in normal serum and performed the precipitation assay as above. The percentage of E. coli 0113 LPS that was precipitated approached that of normal serum at a dilution of 1/32 (data not shown).

Role of Immunoglobulin G in precipitation of heterologous LPS.

To study the role of immunoglobulin in the system, we prepared IgG from normal serum and antisera directed to E. coli J5, E. coli 0113, E. coli 018 and S. typhimurium by anion exchange chromatography. The IgG preparations were then tested for the ability to precipitate radiolabeled LPS in the presence or absence of normal serum (Fig. 4-6). These

experiments revealed that IgG prepared from J5 antiserum was able to precipitate heterologous LPS in the presence of normal serum, but that very large concentrations of immunoglobulin were needed. No precipitation was seen in the absence of normal serum. Immunoglobulin G prepared from antisera directed to smooth organisms precipitated homologous LPS at relatively low concentrations. In sensitive portions of the curve, these immunoglobulins precipitated homologous LPS more efficiently in the presence of normal serum. It required approximately 100-fold more IgG prepared from J5 antiserum than IgG prepared from homologous antiserum to precipitate equivalent amounts of LPS.

Precipitation of heterologous LPS with affinity purified antibody to E. coli J5 LPS.

Affinity purified immunoglobulin prepared on immunoaffinity columns consisting of E. coli J5 LPS coupled to Sepharose 6B was unable to precipitate substantially more LPS in the system, despite approximately equivalent titers of IgG directed to E. coli J5 LPS by ELISA as in the parent antisera (Table V).

CONCLUSIONS

We (27), and others (26), have suggested that sera drawn from rabbits made tolerant to LPS neutralize more LPS than normal serum and that the augmented detoxification is due to increased LPS-lipoprotein binding. Since polyclonal antisera raised to rough mutant organisms are felt to protect against the effects of LPS by increased neutralization of the LPS

(7), and since the role of antibody as opposed to non-antibody inflammatory factors in the protection remains controversial, we compared the LPS-lipoprotein binding in normal serum, tolerant serum, and J5 antiserum. These experiments indicated that radiolabeled LPS incubated in the three sera behave differently, and in particular that LPS incubated in J5 antiserum does not form increased quantities of low density LPS-lipoprotein complexes in the manner that LPS incubated in tolerant serum does.

In the course of these experiments we noted that increased amounts of radiolabeled heterologous LPS were precipitated with calcium and dextran in J5 antiserum compared to normal serum. Further investigation suggested that the increased precipitation in J5 antiserum was due to immunoglobulin G (as prepared by anion exchange chromatography), but that the precipitation required the presence of normal serum to be detectable.

Analysis of immunoglobulin in polyclonal antiserum directed to deep "core" regions of the LPS molecule is complex due to reasons relating to both the antiserum and the antigen. First, the antiserum is generally raised to a killed boiled cell vaccine of a rough mutant organism Consequently, antibodies are induced to many epitopes. Many of these antibodies would recognize epitopes present on the homologous LPS and only a small percentage of these antibodies would be expected to cross-react with heterologous LPS. Therefore, antibody assays utilizing the homologous LPS as antigen, such as E. coli J5 LPS, presumably are mostly measuring antibodies which are not cross-reactive (and therefore not cross-protective). Studies utilizing ELISA and western blotting techniques have shown this to be the case (38, 46). Second, immunization with rough mutant organisms (38, 47) or rough mutant LPS (47-50) results

in nonspecific polyclonal stimulation of B lymphocytes and may increase the antibody directed to the O-polysaccharide chains of heterologous LPS. These mitogenically induced type-specific antibodies confound interpretation of direct binding assays (38). Third, the amphipathic nature of the LPS molecule creates difficulties when it is used as an antigen in most conventional assays of antigen-antibody binding. In solid phase immunoassays such as ELISA the hydrophobic lipid A moiety presumably binds preferentially to the plastic microtiter plate, leading to an altered and artificial presentation of the molecule. A similar situation may exist for LPS in hemagglutination or western blotting assays. Such an altered conformation of the molecule may stereochemically hide cross-reactive epitopes. Furthermore, it is known that the physicochemical configuration of LPS is considerably altered in the presence of serum or plasma. The micelles of LPS that exist in aqueous buffers are disaggregated and then bind to lipoproteins with an alteration in their density (21, 22). It thus seems possible that the epitopes available for binding to immunoglobulin might be different in fluid phase and in the presence of serum than when the LPS is bound to an artificial carrier such as to microtiter plates, red blood cells, or nitrocellulose paper.

The discovery that more radiolabeled LPS precipitates in J5 antiserum than normal serum under conditions designed to precipitate LPS-lipoprotein complexes was unexpected and the molecular basis for this phenomenon is not yet known. Precipitation of LPS-antibody complexes with dextran has been described as a technique for enhancing the precipitation of LPS in immunodiffusion plates (51). Complexes of LPS and type specific

anti-O-polysaccharide antibody were precipitated with calcium and dextran in the absence of serum (Fig. 4-6), suggesting that serum is not essential for precipitation of LPS-antibody complexes to take place. However, at sensitive dilutions of homologous antibody, more LPS was precipitated in the presence of serum. In contrast, essentially no LPS was precipitated by immunoglobulin fractions prepared from normal or J5 antiserum at concentrations greater than one milligram per milliliter in the absence of serum.

A possible explanation of the phenomenon might be that the LPS micelles are disaggregated in serum permitting a more efficient interaction with anti-LPS antibody. It has previously been shown that disaggregation of LPS with triethylamine slightly but significantly increases the amount of anti-O-polysaccharide antibody that binds to LPS in a competitive ELISA system (35). However, this explanation is not sufficient in itself to explain our results because LPS did not precipitate in delipidated J5 antisera despite the fact that it would be disaggregated in these conditions (22, 40). The nature of the complexes that are precipitated are presently being studied. Presumably, they consist of a mixture of LPS-lipoprotein (accounting for the background of 30-50% precipitated in normal sera), LPS-antibody, and perhaps lipoprotein-LPS-antibody. The interactions appear to be complicated. Type-specific immunoglobulin directed to the O-polysaccharide can bind to preformed LPS-lipoprotein complexes (44, 52), but also inhibits the formation of the same complexes if present at the time of the reaction, apparently by preventing the disaggregation of the LPS (52). It is not known if immunoglobulin directed to epitopes in the core region of LPS behave similarly.

Our data do not distinguish between the possibility that the increased precipitation we found in J5 antiserum results from small quantities of anti-O-polysaccharide immunoglobulin that are mitogenically induced by the rough mutant vaccine (38), and the possibility that there are small quantities of cross-reactive IgG in the J5 antiserum which bind to a common epitope on smooth LPS in the presence of normal serum (1). The latter possibility is supported by the results of a recent clinical trial in which the mortality of patients with gram negative bacteremia or shock was decreased by administration of a human monoclonal IgM directed to the core glycolipid (19). Since the cross-reactivity of monoclonal antibodies to LPS has been difficult to assess in solid phase binding assays, a radioimmunoassay such as that described here may he helpful for the analysis or further development of monoclonal antibodies directed to lipid A or to the endotoxin core.

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Table I. Percentage of radiolabeled LPS at density < 1.21 g/cm^3 and precipitated with calcium and dextran after incubation for 30 minutes at 37° C in each serum.

	Percent LPS from *E.coli* 0113 at density < 1.21	precip. in Ca^{++}/Dex	Percent LPS from *S.typhimurium* at density < 1.21	precip. in Ca^{++}/Dex
Normal serum	50.5 ± 4.9	23	24.1 ± 0.2	40
J5 antiserum	47.0 ± 1.4	63	17.7 ± 1.1	62
Tolerant serum	73.5 ± 2.1	62	51.3 ± 0.0	61

Table II. Percentage of radiolabeled LPS that precipitated with Ca^{++}/Dextran after incubation with different sera at 4°C and 37°C.

	E.coli 0113		S.typhimurium	
	4°C	37°C	4°C	37°C
Normal rabbit serum	8	26	4	38
J5 antiserum	12	65	4	59
Tolerant serum	11	57	5	60

Table III. Percent precipitation of radiolabeled E.coli 0113 LPS with calcium/dextran after incubation in normal rabbit serum, polyclonal antiserum directed to E.coli J5, and delipidated antiserum directed to E.coli J5.

	Fresh frozen sera			Delipidated sera ether/butanol			Aerosil		
	Percent precipit.	IgG* titer	IgM* titer	Percent precipit.	IgG* titer	IgM* titer	Percent precipit.	IgG* titer	IgM* titer
Normal Serum	34	0	30	22	ND	ND	16	ND	ND
J5 antiserum	70	15,900	4,300	22	11,500	3,600	20	6,800	3,[illegible]
				ǂ 13	11,100	4,700	ǂ 8	13,500	5,[illegible]

*Directed to E. Coli J5 LPS
ǂConcentrated to IgM antibody titers to E.coli J5 LPS > parent hyperimmune sera.

Table IV. Percent radiolabeled LPS precipitated with calcium/dextran from different rabbit sera after delipidation, tested in saline and in the presence of normal rabbit serum.

Tested with ^{3}H-LPS from	Serum		Not delipidated	Extraction with diisopropyl ether/butanol 75:25	Mixed with Aerosil 45°C, 4 hrs	Density > 1.21 by ultra-centrifugation in KBR
E.coli 0113	Normal serum	undiluted	35	5	7	8
		1:1 with saline		4	12	14
		1:1 with NRS		21	25	22
	E.coli J5 antiserum	undiluted	70	3	5	15
		1:1 with saline		3	5	18
		1:1 with NRS		62	63	62
S.typhimurium	Normal serum	undiluted	30	5	11	13
		1:1 with saline		5	10	14
		1:1 with NRS		18	33	31
	E.coli J5 antiserum	undiluted	66	3	6	34
		1:1 with saline		7	5	23
		1:1 with NRS		38	51	51
E.coli 018	Normal serum	undiluted	51	4	10	9
		1:1 with saline		4	9	12
		1:1 with NRS		33	47	40
	E.coli J5 antiserum	undiluted	84	5	8	25
		1:1 with saline		6	6	16
		1:1 with NRS		73	81	74

Table V. Percent precipitation of radiolabeled LPS by calcium/dextran in normal rabbit serum spiked with saline or affinity purified anti-E.coli J5 antibody.

^{3}H-LPS	Serum + saline (1:1)	Serum + anti-J5 Ab* (1:1)
E.coli 0113	21	16
E.coli 018	46	42
S.typhimurium	35	43

*Antibody titer directed to E.coli J5 LPS in affinity purified sample was 10,100 for IgG and 900 for IgM by ELISA. Antibody titer in parent antiserum was 12,000 for IgG and 2,000 for IgM.

LEGENDS TO FIGURES

Figure 1. Percent E. coli 0113 LPS precipitated in normal serum (▲—▲) and J5 antiserum (△···△) as a function of incubation time at 37° in minutes.

Figure 2. Percent S. typhimurium LPS precipitated in dilutions of normal serum (▲—▲), J5 antiserum (△···△) and tolerant serum (◆—◆). Similar results were obtained with LPS from E. coli 0113. The second peak at dilutions 1/32-1/256 was consistently present for each serum tested.

Figure 3. Percent E. coli 0113 LPS precipitated after 30 min in delipidated normal serum (▲—▲) and delipidated J5 antiserum (△···△) as a function of the percentage of normal serum present in the reaction mixture. Concentrations of delipidated sera (50% of reaction mixture) were identical for all points. The two isolated points plotted at lower left of figure represent delipidated normal serum (●) and J5 antiserum (✱) combined 1:1 with saline alone.

Figure 4. Percent E. coli 0113 LPS precipitated by normal serum combined 1:1 with IgG purified from antiserum directed to E. coli 0113 (□—□), E. coli J5 (△···△), S. typhimurium (○—○) and normal serum (▲—▲). Also plotted is percent E. coli 0113 LPS precipitated by normal saline combined 1:1 with IgG purified from antisera directed to E. coli 0113 (□···□) and E. coli J5 (single point *). Antibody concentrations plotted represent final protein concentrations of reaction mixture. Antibody titers for each IgG fraction are expressed relative to 4.0 mg/ml protein. Titers of each IgG to its

homologous LPS by ELISA were 192,000, 7,800, and 2,800 for E. coli 0113, E. coli J5, and S. typhimurium respectively. ELISA titers in the IgG fraction from normal serum directed to LPS from E. coli 0113, E. coli J5, E. coli 018, and S. typhimurium were 41, < 25, 16, and 83 respectively. ELISA titers in the IgG fraction from J5 antisera directed to heterologous LPS from E. coli 0113, E. coli 018, and S. typhimurium were 85, 14, and 10 respectively.

Figure 5. Percent S. typhimurium LPS precipitated by normal serum combined 1:1 with IgG purified from antiserum directed to S. typhimurium (O—O), E. coli J5 (Δ···Δ), E. coli 0113 (□—□) and normal serum (▲—▲). Also plotted is percent S. typhimurium LPS precipitated by normal saline combined with 1:1 with IgG purified from S. typhimurium (O--O) and E. coli J5 (single point *). Antibody concentrations plotted represent final concentrations of reaction mixture. Antibody titers are given in legend to Fig. 4.

Figure 6. Percent E. coli 018 LPS precipitated by normal serum combined 1:1 with IgG purified from antiserum directed to E. coli 018 (◎—◎), E. coli J5 (Δ···Δ) and normal serum (▲—▲). Antibody concentrations plotted represent final concentrations of reaction mixture. The ELISA titer of IgG purified from antiserum directed to E. coli 018 was 511,000 when tested with 4.0 mg/ml protein. The antibody titers for the other IgG fractions are given in the legend to Fig. 4.

Fig. 1

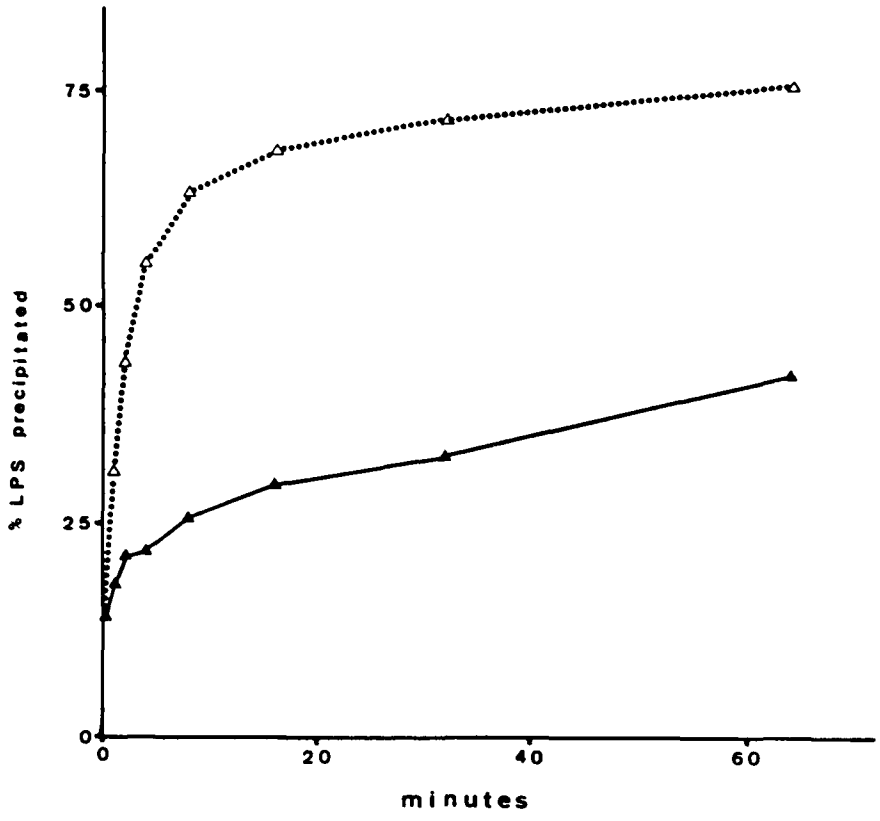

% LPS precipitated
75
50
25
0
0
20
40
60
minutes

Fig. 2

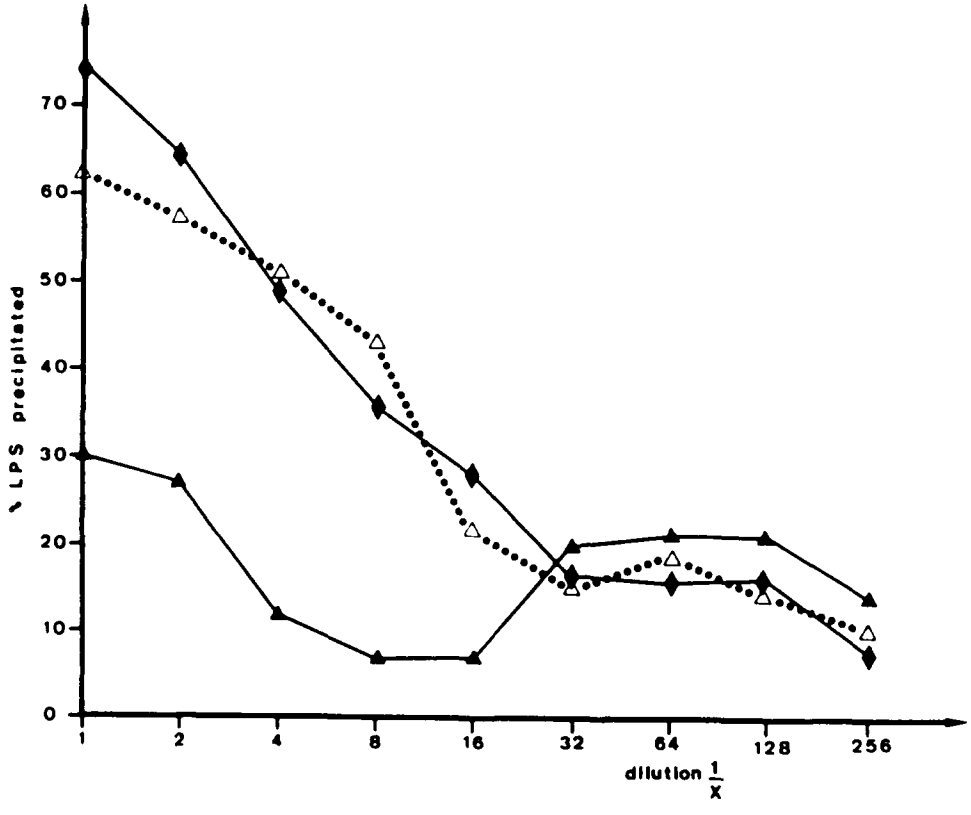

% LPS precipitated
70
60
50
40
30
20
10
0
1
2
4
8
16
32
64
128
256
dilution 1/x

Fig. 3

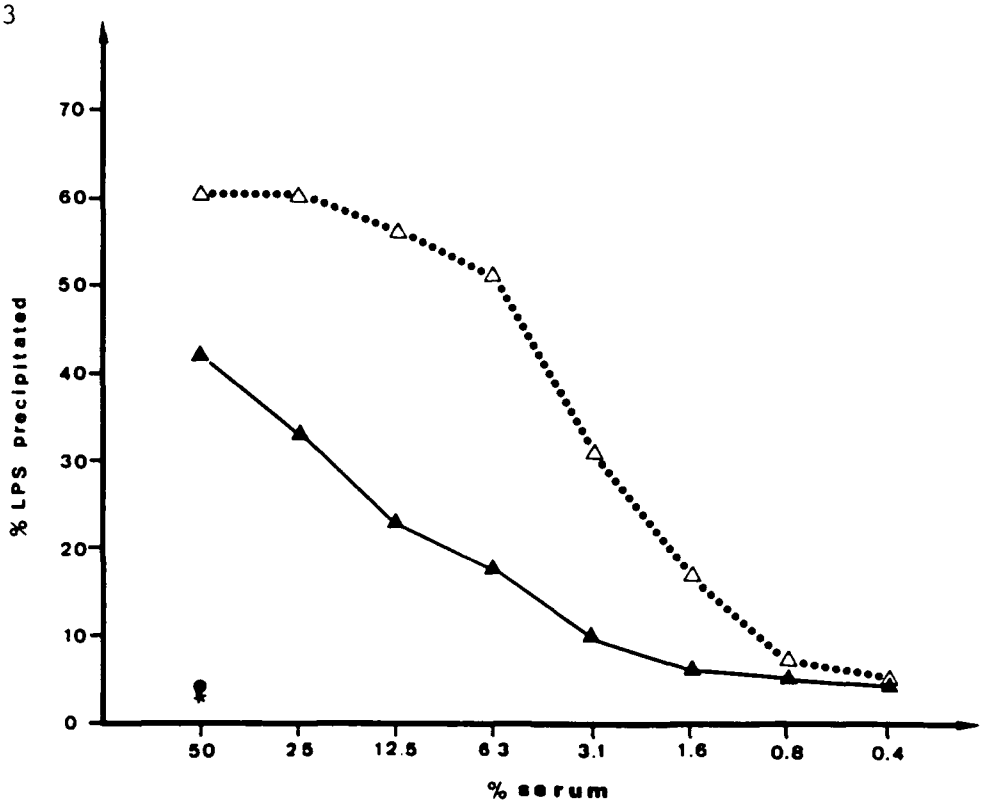

70
60
50
40
30
20
10
0
% LPS precipitated
50
25
12.5
6.3
3.1
1.6
0.8
0.4
% serum

Fig. 4

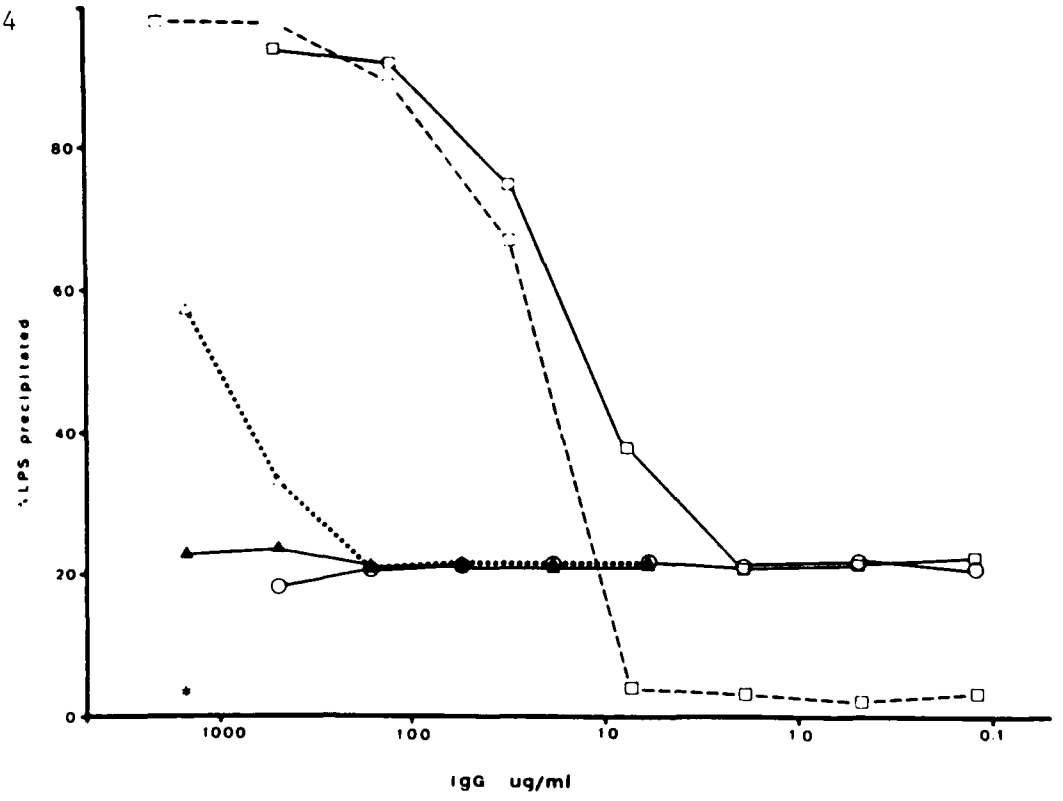

80
60
40
20
0
% LPS precipitated
1000
100
10
1.0
0.1
IgG ug/ml

Fig. 5

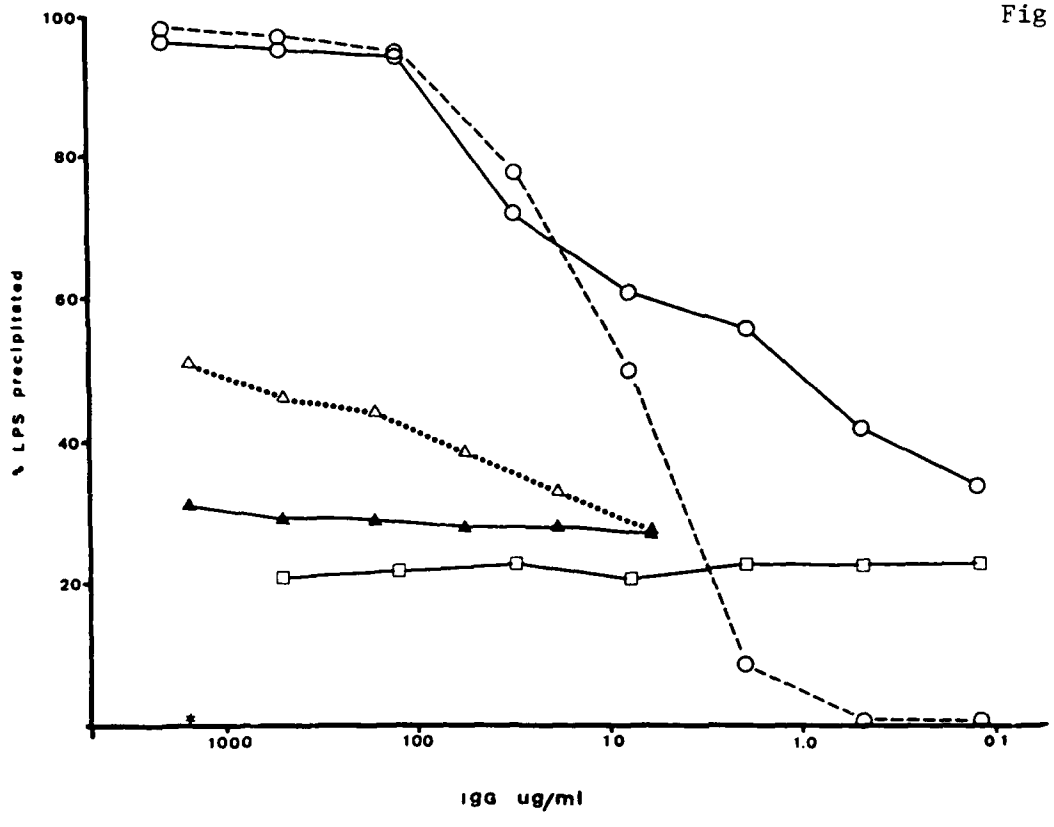
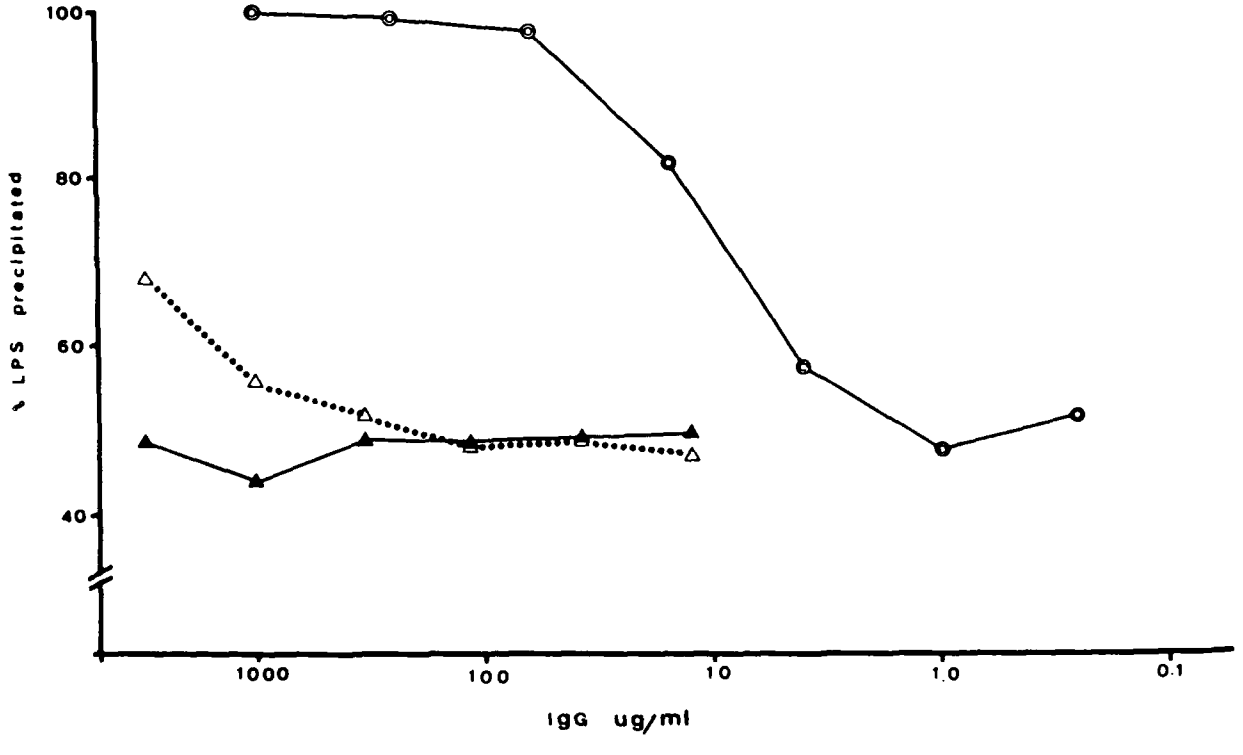

100
80
60
40
20
% LPS precipitated
1000
100
10
1.0
0.1
IgG ug/ml

Fig. 6


100
80
60
40
% LPS precipitated
1000
100
10
1.0
0.1
IgG ug/ml

IN PRESS (Current Clinical topics of Infectious Disease)

Infection of Burn Wounds: Evaluation and Management

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Introduction

Infection plays a major role in the morbidity and mortality of burns. A study in 1980 indicated that greater than 50% of deaths in patients with burn injuries were due to complications of sepsis (1). Amongst other adverse effects, infections also delay burn healing, contribute to graft failure, and convert second degree burns to full thickness third degree burns.

There are multiple reasons for the frequency and severity of infections in patients with burn wounds. First, the barrier function of the skin is lost, allowing microbial access to underlying tissues. A similar process takes place in the pharynx and respiratory tree following inhalation injury. Second, the serosanguinous fluid exudate that occurs in response to skin loss serves as an excellent culture medium. Third, the immune system is profoundly impaired in patients with severe burns (reviewed in 2,3). Serum immunoglobulin (4,5) and complement (2,6) are decreased, chemotaxis of neutrophils is depressed (7,8), and cell-mediated immunity is diminished (2,3,9). The specific etiology of each deficit is not well understood, and is felt to be multifactorial and related to the induction of massive inflammation caused by the burn wound itself,

protein loss from the skin surface, and interrupted synthesis of necessary substrates due to the catabolic state. Burn patients are often anergic. Allograft rejection is delayed, and functional tests for classical and alternative complement function are markedly abnormal (2,3,5,6). Finally, the need for intensive and aggressive support often mandates the prolonged use of intravenous, intra-arterial and foley catheters and endotracheal tubes which are positioned adjacent or through heavily colonized or infected skin surfaces. Infectious complications relating to these foreign bodies are common.

Patients with burn wounds pose a particular challenge to the Infectious Disease consultant. The physical exam is made difficult by extensive dressings and isolation procedures. Clinical and laboratory indications of infection such as fever and leukocytosis are obscured by underlying inflammation caused by the burn itself. Interpretation of culture data is complicated by the difficulty of obtaining appropriate samples from patients who have extensive microbial colonization in open burn wounds. A comprehensive review on the overall care of patients with burns has recently been published (10). This chapter will attempt to present an approach to the evaluation and management of infection. It should be noted that there is a lack of definitive data on many specific issues, and that therefore much of the treatment of these infections is empirical, albeit based upon logical assumptions.

Need for a coordinated systematic approach to treatment

The management of patients with severe burn wounds differs from that of patients with most other injuries in several aspects which will be addressed in the following sections. One of the more important differences is that the hospital course of these patients is somewhat predictable. It is usually clear at admission that the patient will be acutely ill for a relatively long period (weeks to months) that extends from the time of the burn to the time of total closure of the wounds. The extended treatment period leads to predictable problems (such as repeated infections). It is helpful to develop a unified and coordinated treatment plan at admission for the entire illness in order to anticipate and optimally manage such problems.

The prevention and care of burn wound infection is an important part of this overall plan. The nursing service plays a pivotal role in this regard. The spread of infection is decreased by compulsive nursing care, both through the careful application of dressings and by adhering to strict infection control techniques. Since extensive dressings often hinder daily examination of every wound surface, the burn patient's nurse is also often the best source of information pertaining to which wounds might need attention.

The knowledge that a patient with a serious burn will likely have open wound surfaces and repeated infections for weeks to months may affect decisions concerning the initiation, choice, and duration of antimicrobial agents. The treatment period is usually accompanied by the steady

emergence of increasingly resistant colonizing microorganisms. Over aggressive use of antibiotics early in the course of the illness hastens the appearance of microbial resistance which then may become problematic later in the illness. Accordingly, it is prudent to limit the use of antibiotics as much as possible, and when needed to utilize agents with a narrow spectrum for as short a time as is deemed sufficient.

Early Evaluation

Burns are classified by the extent of the injury (estimated as a percentage of total body surface area involved) and by the depth of the injury. The exact source of the energy causing a thermal burn (i.e., scald, flame, flash explosion, etc.) is ultimately of lesser importance, except as it relates to secondary problems such as smoke or vapor inhalation. The depth of a burn is estimated by physical examination and the appearance of the burn over several days. Depth is described in terms of degrees (from first to fourth) utilizing skin structures as landmarks (Fig. 1). First degree burns involve the superficial epithelium only. They are dry, red, and painful to touch and are typlified by sunburn. Second degree burns involve the epidermis and part of the dermis, but spare deep structures such as hair follicles and sweat glands. These burns are wet, erythematous, painful to touch, and often blistered. Third degree burns involve the full thickness of the skin and thus include the epidermis, dermis, and hypodermis. They may be white or charred or dark red from fixed hemoglobin. If red, they do not blanch. Third degree burns are painless to the touch. Hair is not present or is easily extracted.

Extremely deep burns which involve structures beneath the skin such as muscle or bone are sometimes referred to as fourth degree burns.

Chemical and electrical burn injuries are associated with some special problems. Chemical injuries are similar in appearance to thermal injuries and carry a similar risk of infection. However, many chemical agents which cause burns produce tissue injury that continues long after the first exposure. An extreme example of this is hydrofluric acid. Removal of the chemical agent from the tissue by extensive and prolonged washing is therefore very important. Electrical burns caused by high voltage are devastating injuries with considerable deep tissue destruction. The entrance wound is usually a small black charred area. Its size and unimpressive appearance is sometimes misleading. Deep muscle necrosis may extend for large distances, especially in the extremities. The muscle injury may be indolent and therefore unrecognized, but may be a nidus for severe and life threatening infection in the second week (or later) of the illness.

The initial evaluation should attempt to assess whether there has been damage to the tracheobronchial mucosa from inhalation of heat or toxins within smoke. This is sometimes difficult because mucosal edema may not appear for 24-48 hours. Clues can be obtained from the nature of the burn, the location where the burn occured (open vs. closed space), and the presence of burns in the pharynx or hypopharynx or carbon in the sputum. Bronchoscopy is often helpful. Inhalation of heat or toxins increase the likelihood of pneumonia by directly damaging the tracheal or bronchial

mucosa, by providing bronchial and alveolar fluid in which microorganisms can grow, and by decreasing the ciliary clearance of bacteria. Corticosteriods given for 72 hours following admission in an attempt to prevent inflammation in the lung have been shown to have no effect on pulmonary complications, and are associated with increased infections (11,12). Areas of special concern are the eyes and the ears in patients with facial burns. If there is any possibility of corneal damage an ophthalmologist should be called to perform a detailed exam. Exposed cartilage in the ears (and elsewhere) is at risk for secondary suppurative chondritis.

Several prophylactic issues relevant to patients with burns need to be addressed early in the management. Burn wounds are susceptible to infection with C. tetani. Accordingly, it is appropriate to administer a tetanus toxoid booster in all patients without a recent history of immunization. In addition, human tetanus immune globulin (TIG) should be given intramuscularly in patients who have dirty wounds which are obviously contaminated and in which the risk of tetanus seems high, or in patients with an unclear history of primary immunization against tetanus. A second, controversial issue is the question of whether to administer prophylactic penicillin G in order to decrease the frequency of early Group A streptococcal sepsis. The use of prophylactic penicillin emerged in the early antibiotic era when Group A streptococcus was the major pathogen in burns. Rapidly progressing sepsis with shock soon after the burn injury was common. Today, serious Group A streptococcal infections in burn patients are relatively rare. A small prospective placebo

controlled study in 1982 concluded that prophylactic penicillin is not indicated, but the number of patients was small (fifty-one) and there was only one episode of streptococcal bacteremia (13). Because of the present low incidence of serious streptococcal infections, a large study would be needed to address the risk/benefit ratio of prophylactic penicillin administration in this patient population. Many centers no longer prescribe pendicillin G on a routine prophylactic basis. Some centers administer prophylactic penicillin to children but not adults, reasoning that colonization of Group A streptococcus may be higher in children than adults.

The use of preventive (prophylactic) topical antimicrobial agents, which are usually started immediately after the burn injury, will be addressed in a separate section.

Microbiology of Burn Wounds

The microbiology of burn wounds not unexpectedly reflects the microbiology of the hospital environment and consequently varies between hospitals and burn centers. Several decades ago, gram positive organisms were the predominant pathogens. Currently, gram negative organisms, such as Pseudomonas species, are a more frequent problem. Because the surface area available for colonization supports a high bacterial burden, a major difficulty is the selection of drug resistent organisms by repeated courses of antibiotics. The large surface area of wound which is open for

weeks or months and the requirement for intensive nursing also increase the liklihood of the nosocomial acquisition of problem pathogens such as methicillin resistent S. aureus (MRSA) or resistent gram negative bacilli from other hospital patients. Repetitive treatment with antibiotics frequently leads to fungal colonization of wounds, uninvolved skin, the oral cavity, and stool (14,15).

One of the most difficult problems in the care of burn patients is the interpretation of culture data. Since burn eschars are universally and heavily colonized, surface cultures are not helpful in distinguishing infection from colonization. Different culture methods have been tried for decades in the hope of resolving this problem. Early swabbing techniques (16,17) led to the concept of full thickness biopsies in order to obtain quantitative cultures (18,19). It was proposed through a process of trial and error that colony counts of greater than 10^{4-5}/gram of biopsy tissue correlate highly with burn wound infections (20, reviewed in 21). With this technique, the biopsy is simutaneously cultured, processed histologically, and stained for microorganisms. The value of this approach is unclear. Paired samples vary substantially, raising doubts as to the validity of single biopsy. In addition, a relatively recent study indicated that there was histological evidence of infection in only a minority of samples which had greater than 10^5 organisms/gram (22). A final difficulty is that the biopsy technique is too time consuming and expensive for routine use.

There are no data on the value of surveillance cultures in patients with burns, and there is therefore little concensus as to their utility. Many authors on the subject suggest culturing wounds (with swabs), urine, and respiratory secretions at some interval in order to acquire knowledge of problem pathogens and antibiotic resistant patterns before needing to treat them. At the Massachusetts General Hospital Burn Center and the Shriners Burns Institute in Boston this is done weekly. Our impression is that this information is helpful in choosing an initial antibiotic regiment in the face of sepsis. Attention is directed in particular to changes in the microbial species and estimated concentrations (few vs. heavy) of colonizing organisms. Some authors have recommended a very aggressive approach such as performing quantitative biopsy cultures every 48 hours (20). The possible benefit of surveillance cultures need to be balanced against the labor and cost required.

Topical Antimicrobial Therapy

It is generally assumed that the use of topical antibiotics have contributed to the decrease in mortality in burn care over the last 20 years. There are however no controlled trials proving their efficacy. The rationale of the use of topical antimicrobial agents is to slow the aquisition of burn eschar colonization, and diminish its extent, thereby decreasing the incidence of invasive burn wound infections. Topical antibiotics are thus felt to be most helpful early in the course of the burn. Their use is the subject of a recent review (23).

The most widely used topical antimicrobial is silver sulphadiazine, which is prepared by reacting silver nitrate with sodium sulphadiazine to from an insoluble white compound. It is applied twice daily in a 1% water soluble cream. This layer of cream needs to be removed before successive treatments in order to be maximally effective. Silver sulphadiazine has a very wide antimicrobial spectrum, although little is known definitively about its mechanism(s) of action and the respective roles of the silver and sulpha components. It is minimally absorbed into the eschar, although there is some absorption of sulpha into the bloodstream. Side effects are similar to those of sulphadiazine. Plasmid mediated resistance to both silver (24,25,26) and sulphadiazine (27) have been reported, yet widespread resistance has not been a problem so far, presumably because the plasmids have been unstable.

Another effective antimicrobial with which there is a 25 year experience in burn wounds is dilute silver nitrate (28). Its main advantages are its extremely broad spectrum, the relatively rare selection of resistant organisms, and the fact that it is painless. Aqueous $AgNO_3$ (0.5%) is applied to the wound in a thick wet dressing covered with a dry surface dressing. This arrangement is comfortable, allows free joint movement, and provides an excellent barrier against evaporative heat and fluid losses. There are however several disadvantages to its use. Its solubility characteristics require that it be dissolved in distilled water. In order to avoid evaporation it needs to be soaked in bulky dressings which must be wetted every two hours. This is inconvenient, and in large burns the copious quantities of hypotonic solution can lead to a

significant electrolyte loss. Penetration of silver nitrate into the eschar is minimal, and the solution stains tissue, bedding, and floors black if it is used carelessly and splashed on surfaces. A rare complication is methemaglobinemia from absorption of nitrites produced by reduction of nitrate by bacteria in the eschar. Finally, silver nitrate should be used with caution on the face where it can irritate the eyes with continuous use or be ingested.

A topical agent that was used extensively in the past is a methylated sulfonamide called mafenide (alpha-amino-P-tolulene sulfonamide monoacetate), (Sulphamylon^{R}). It has a wide antimicrobial spectrum against gram positive and gram negative organisms, including Clostrida, and rapidly penetrates the wound eschar. An important side effect however is that mafenide is a strong carbonic anhydrase inhibitor and can lead to a severe metabolic acidosis. Other described disadvantages are considerable pain on application to second degree burns, the emergence of resistance, and allergic side effects related to the sulpha component. Since mafenide rapidly and effectively penetrates the burn eschar, it is sometimes liberally applied to a heavily colonized burn eschar 4-6 hours before surgical excision in order to diminish the bacterial burden preoperatively.

Less commonly used preparations include iodophors and preparations containing bacitracin (either alone or with other antibiotics such as neosporin and colistin). The iodophors have the disadvantage of iodine absorption, poor penetration of tissues, and staining, whereas bacitracin

alone has a limited antibacterial spectrum. The risk of systemic absorption of bacitracin/neosporin/colistin also limits its use to relatively small surface areas.

There are no prospective studies that are helpful in determining which topical antimicrobial might be preferable. In 1976, Feller et al reviewed data from patients registered with the National Burn Information Exchange and found no difference in survival curves between large groups of patients treated with either sulphadiazine, silver nitrate, or mafenide (29). In fact, there was little difference between these groups and patients who had received no topical antimicrobials at all. It is, however, difficult to draw a definitive conclusion from this study because it is a retrospective analysis of a large pool of data that was obtained from different sources.

Similarly, there are no data to provide guidance as to the value of topical antibiotics for protecting the eyes against invasive infection. These are perhaps best avoided unless the burn wound either involves or is directly contiguous to the eye and there is evidence of infection. Although optic solutions of antibiotics including erythromycin, neomycin, polymyxin, and sulphacetamide are sometimes utilized in the setting of facial burns, simple ophthalogic lubricating solutions or ointments are probably preferable in the absence of documented infections. If there is a question of infection, appropriate stains and cultures should be obtained from the conjunctiva to help guide therapy. Deep infections should be managed with the help of an ophthalmologist.

Role of Isolation of Burn Patients

Isolation of patients with burns has been advocated as a means of decreasing the acquisition of hospital pathogens (30,31). There has been only one large study on the issue which was published in 1977 (30). For this study special environmental units surrounding the patients' bedside were constructed using clear plastic walls and sterilized unidirectional air flow from ceiling to floor. Patients were treated in these units or on open acute care wards, and an attempt was made to document whether new organisms colonizing burn wounds came from the patients' indiginous flora, defined as being present upon surveillance cultures on admission, or from another source. Patients in the environmentally controlled units had more severe burns, but less colonization with extrinsic hospital organisms compared to patients on the open ward. In both groups new colonization with indiginous microorganisms from a different body site was much more frequent than wound colonization with extrinsic hospital organisms. In this study, the acquisition of extrinsic hospital pathogens into burn wounds by patients in the environmentally controlled isolation units was more likely to be associated with clinically defined infections when compared with the acquisition of indiginous bacteria from another body site (65% vs 39%, $p < .05$).

The lack of data does not permit a conclusion as to the need for strict isolation. It seems reasonable to attempt to minimize the aquisition of new potentially resistent hospital pathogens, and the available evidence suggests that this is possible using environmentally

isolated units and rigorous attention to their proper use. Whether the extra effort is justified, however, has not yet been solidly proven. Hopefully, the use of "universal precautions," which have recently been implemented in response to the epidemic of human immunodeficiency virus, will also lead to a decrease in the incidence of the cross-contamination of burn wounds.

Surgical Issues

1. Early burn wound excision and grafting

Controversy has existed for many decades as to the role and timing of surgery in burn wounds. In the 1960's most wounds were treated non-operatively. Infectious complications from heavily colonized burn eschars were a large problem despite the use of topical antibiotics. In the late 1960's and early 1970's, the use of surgical excision of burn eschars and immediate wound closure became a routine treatment of deep burn injury. This system utilized increasingly aggressive surgery to excise the burn wound within the first week of the initial injury in patients with deep dermal and full thickness burns. The resultant wound was then grafted. The onset of this approach, referred to as "early" or "prompt" excision, correlated temporally with a decrease in sepsis, length of hospital stay, and mortality (32-37), and has now been adopted by most burn centers. A retrospective study published in 1983 compared 11 specialized burn centers and found that mortality in burn injuries is

inversely proportional to the mean institutional speed of wound closure (38). A major benefit of early excision is felt to be reduction of infection. This seems likely, given the progress that has been made. Nonetheless, there has unfortunately been only one randomized prospective trial studying early excision (37), and it involved only small numbers of patients with burns of indeterminant depth that were less than 20% of the total body surface area. Survival in burn patients has improved considerably over the last 15 years. Although the reasons for this are multifactorial, there is wide agreement that the routine use of early burn wound excision has played an important role.

2. Wound Closure Materials

The use of early burn wound excision has created a need for wound closure materials. Burns of less than 35% to 40% of the total body surface area are usually covered with split thickness autografts. Larger burns require exogenous materials as temporary skin substitutes. Possibilities include allografts (cadaver or living-related donor), xenografts, temporary synthetic skin substitutes, and materials leading to biologic-skin replacement (e.g., epidermal cells, artificial skin). All of these materials, except those that lead to biologic skin replacement, are temporary but provide at least short-term protection against burn wound infection. The materials leading to biologic skin replacement show considerable promise, but have not been used long enough to provide a complete view of their efficacy.

3. Infections of graft donor sites

Split thickness graft donor sites are covered either with an adherent dressing that is incorporated into the dried exudate and which is eventually sloughed, or with a non-adherent dressing which can be removed at any time. Infections of these sites are rare, but occur, and have the potential of turning the donor site into a full thickness injury. Infected donor sites are treated as partial or full thickness burns.

Systemic Antimicrobial Therapy

Assessment of whether a burn wound *is infected (as opposed to* colonized), and consequently whether systemic antibiotics should be used, is often difficult. Most patients with large burns have fever and leukocytosis caused by inflammation induced by the burn injury itself, and cultures of the burn wound are uniformly positive. Moderate fever in the range of 100°F to 102°F may persist for weeks or even months into the hospital course without an identifiable infection, especially if there are uncovered wounds. A temptation to utilize systemic antibiotics early needs to be tempered with the understanding that selection of highly resistent organisms is common in patients with extensive burns and that the likelihood of future infection with these organisms is relatively high. Helpful clues to infection include increased purulent drainage (over and above the wound exudate), and indications of systemic sepsis (eg. metabolic acidosis, hypotension, decreased urine output, alterations

in fever or leukocytosis from a previously established baseline, altered CNS or gut function, hyperglycemia, etc). Blood cultures are frequently difficult to interpret. The chance of contamination of blood cultures with likely pathogens is increased because of the lack of easy sterile access to the bloodstream. If a blood culture is positive, it is sometimes impossible to distinguish between contamination, an infected intravascular catheter, and burn wound infection with bacteremia. Frequently it is necessary to empirically treat the patient with systemic antimicrobials based upon clinical signs of sepsis. It seems logical to base the choice of such empiric treatment on surveillance and wound cultures until the results of blood cultures are known. Often this will include an aminoglycoside together with a beta lactam antibiotic or vancomycin. Aminoglycosides penetrate well into the burn eschar (39,40). The antibiotics can then be tailored to <u>in vitro</u> sensitivities if the blood cultures are positive, or stopped if the blood cultures are negative.

Dosing of antimicrobials in patients with burns needs to be carefully monitored. The half-life of aminoglycosides is erratic and shortened in children (41) and adults (42,43) with burns, presumeably due to high fluid losses from wound exudates and an increased glomerular filtration rate (43). Accordingly, larger doses than usual are often needed to achieve appropriate serum levels. This is probably also true for many other antimicrobial agents (43,44). On the other hand, some patients also have hepatic and/or renal dysfunction due to multi-organ system failure. It is therefore especially important to measure serum antibiotic levels when possible.

Many burn centers, including our own, utilize preventive (prophylactic) antibiotics tailored to wound surveillance cultures for 8-24 hours beginning with anesthesia and surgical debridement of burn wounds. This practice is based upon the impression that surgical manipulation may lead to endotoxemia and/or bacteremia with sepsis and the risk of metastatic infection. There are no clinical trials that address the efficacy of this practice or the use of prophylactic antibiotics for other aspects of burn wound care except as noted above.

Diagnosis and Treatment of Secondary Infections

Secondary infections are common in burn patients and are treated as in any other patient population. The diagnosis of these infections is complicated by the burn itself. Pneumonia is a particular problem and is probably the most frequent secondary infection encountered. Differentation of pulmonary infection from Adult Respiratory Distress Syndrome (ARDS) induced by smoke inhalation is difficult (45). The single most helpful test probably is the sputum gram stain. For reasons that are not well understood, large and life-threatening pulmonary emboli are unusual despite the prolonged hospital course in which the patient is immobilized. Sepsis due to infected catheters is common. Suppurative thrombophebitis arising from indwelling intravenous catheters was first described in burn patients in 1970 (46) and continues to be a problem. Diagnosis requires a high index of suspicion. These infections are best avoided by the frequent changing of central and peripheral intravenous

catheters. Other secondary infections that occur not infrequently and which may be initially overlooked are endocarditis (47), sinusitis, cystitis, and osteomyelitis. The diagnosis of osteomyelitis is complicated by inflammation from the burn wound itself which may lead to periosteal elevation on radiographs and a positive bone scan in the absence of true infection. A bone biopsy may be necessary to provide a tissue and microbiological diagnosis. Secondary infections with fungi (15), especially Candida species (14), may require prolonged antifungal therapy because the underlying immunosuppression makes dissemination more likely than in patients who are immunocompetent. Although each case needs to be individually evaluated, the presence of extensive fungal colonization ordinarily does not mandate antifungal therapy. On the other hand a single positive blood culture for yeast is probably sufficient grounds for empirically treating the patient with Amphotericin B while awaiting further blood cultures. Disseminated herpes simplex virus infections in this patient population have been described (48). Finally, as in any immunosuppressed group of patients, the possibility of the reactivation of latent infections (eg M. tuberculosis) should be kept in mind.

Conclusion

The importance of infections in the outcome of burn wounds cannot be overemphasized. Indeed the treatment of patients with burns consists, to some extent, of a race to cover burn wounds before recurrent infections

can no longer be successfully treated. In this chapter we have attempted to summarize our approach and review the literature with respect to these infections. Substantial progress has been made over the last several decades, and the progressive decrease in mortality over this time period is gratifying. It should be apparent however that much of the approach to infections in burn patients is empiric. There is a need for carefully performed prospective studies on infectious disease issues in burn patients in order to provide future therapeutic guidelines.

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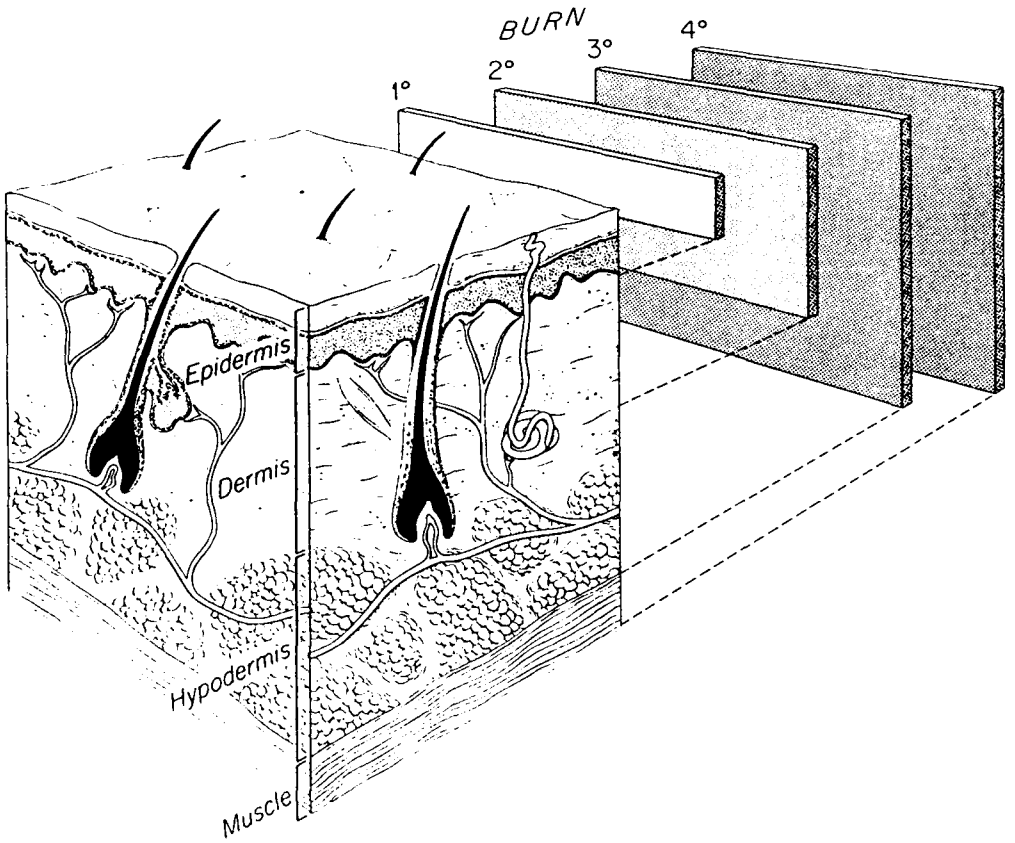

Figure 1. Schematic drawing of skin tissue illustrating lowermost involvement of different degrees of burns (from Tompkins RG, Burke JF. Burn Wound. IN: Cameron JL, ed. Current Surgical Therapy: 3rd ed. Toronto: BC Decker, 1989:696.)